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Novel cysteine-rich secretory protein in the buccal gland secretion of the parasitic lamprey, *Lethenteron japonicum*

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Abstract

Lampreys are one of the most primitive vertebrates diverged some 500 million years ago. It has long been known that parasitic lampreys secrete anticoagulant from their buccal glands and prevent blood coagulation of host fishes. We found two major protein components of 160 and 26 kDa in the buccal gland secretion of parasitic river lamprey, *Lethenteron japonicum*. The larger protein was identified as river lamprey plasma albumin. The complete primary structure of the 26-kDa protein was determined by protein and cDNA analysis. It belonged to the cysteine-rich secretory protein (CRISP) superfamily that includes recently identified reptile venom ion-channel blockers. Lamprey CRISP blocked depolarization-induced contraction of rat-tail arterial smooth muscle, but showed no effect on caffeine-induced contraction. The result suggests that lamprey CRISP is an L-type Ca^{2+}-channel blocker and may act as a vasodilator, which facilitates the parasite to feed on the host's blood. The lamprey CRISP protein contains a number of short insertions throughout the sequence, when aligned with reptilian venom CRISP proteins, probably due to the large evolutionary distance between the Agnatha and the Reptilia, and may represent a novel class of venom CRISP family proteins.

Keywords: Lamprey; Buccal gland; Plasma albumin; CRISP; Primary structure; Smooth muscle contraction; Calcium channel; Calcium channel inhibitor; Cysteine-rich secretory protein; Toxin; Venom; Protein purification; Molecular evolution; Blood coagulation; Parasitism

Lampreys, together with hagfishes, are the most primitive vertebrates diverged at the Ordovician period, about 500 million years ago [1]. Recent fossil evidence indicated that lampreys in the late Devonian period already possessed major characteristics of the present day lampreys [2]: Their morphology, and probably life style as well, have been quite stably conserved over 360 million years. Thirty-eight species are currently recognized for the extant lampreys, of which 16 species are parasitic at the feeding stage in their life cycle [1,3,4]. Although numerous parasitic invertebrates are known, vertebrate parasites are quite rare, and only lampreys and vampire bats are true ectoparasites [5]. Lampreys attach to the host fish for days and secrete anticoagulant called lamphredin from their paired buccal glands to prevent clotting of the host's blood [6]. In this respect they resemble hematophagous leeches, which secrete hirudin and many other anticoagulant proteins [7]. In contrast to the extensive studies on the leech anticoagulants, biochemical nature of lamphredin has long been left uninvestigated since the discovery of anticoagulant and hemolytic activities in 1927 [6]. Very recently, fibrinogenolytic activity of a 160-kDa protein in the secretion of

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Lamepetra japonica was reported [8], but detailed biochemical study is still lacking. We have been investigating the buccal gland secretion of Japanese river lamprey, *Lethenteron japonicum* (a synonym for *Lampetra japonica* [4]), for protein components with anticoagulant and other biological activities. The present paper describes purification and identification of two major components in the buccal gland secretion, lamprey plasma albumin and a novel cysteine-rich secretory protein (CRISP). Ion channel-blocking activity of the latter is also demonstrated.

Materials and methods

Materials. Spawning lampreys were collected at the Shinano River Ohkouzu Floodway and the Miomote River, Niigata, Japan in January and June. Content of the buccal glands was withdrawn by a syringe and stored frozen until use.

Fractionation of buccal gland secretion. Buccal gland secretion from 2 individuals was diluted 5-fold with 20 mM Tris–HCl buffer/0.1 M NaCl (pH 8) and centrifuged. The supernatant was loaded to a Sephacryl S100 column (GE Healthcare Bio-Science Corporation) equilibrated and eluted with the same buffer. Elution was monitored by absorbance at 280 nm. Fractions containing proteins were pooled and further purified by gel filtration on a Superdex 75 HR column (GE Healthcare Bio-Science Corporation) or reversed-phase HPLC on an octylsilane column (Capcell Pak C8, Shiseido).

Analysis of amino acid composition. Protein and peptide samples were hydrolyzed with vapor of 5.7 M HCl for 22 h at 110 °C in a Pico-Tag workstation (Waters Associates) under reduced pressure. Amino acid composition was analyzed by a Hitachi 835 amino acid analyzer. Amino acid analysis was also used to quantify proteins.

Amino acid sequence analysis. Proteins were reduced and *S*-pyridylethylated [9] and digested with lysyl endopeptidase (*Achromobacter* protease I, Wako Pure Chemicals). Peptides were separated on an octylsilane column equilibrated with 0.05% trifluoroacetic acid and eluted using a linear gradient of acetonitrile concentration to 75%. Amino acid sequence was analyzed by an Applied Biosystems 476A protein sequencer.

Mass spectrometry. Molecular mass of protein was determined by MALDI-TOF-MS (Axima CFR, linear mode, Shimadzu Biotech) using sinapic acid as matrix.

cDNA cloning and sequencing. Total RNA was purified from buccal glands by RNAwiz (Ambion, Inc.) as recommended by the manufacturer. First strand cDNA was synthesized with oligo(dT) using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Bio-Science Corporation). A degenerated forward primer 5′-ACS TCS GTS AAC GAC TGG AAG-3′ corresponding to the amino-terminal sequence TSVNDWK and a reverse one 5′-SGT GCA SAG GTT GTT GTC G-3′ corresponding to one of the lysyl endopeptidase peptide CDNNLCT were used for PCR amplification of the coding sequence (S, either G or C). The sequence was extended to the 3′-end by a forward primer 5′-TC AAC AAA CCC TAC GAC CTG G-3′ designed on the basis of the nucleotide sequence for INKPYDLG (residues 136–173). The sequence of the cDNA 5′-end was determined by 5′-rapid amplification of cDNA end (5′-RACE) using SMART cDNA Library Construction Kit (Clontech Laboratories, Inc.). Nucleotide sequences were determined by a DNA sequencer (CEQ 8000 Genetic Analysis System, Beckman-Coulter) using Zero Blunt PCR Cloning Kit for Sequencing (Invitrogen).

Preparation of rat-tail arterial muscle strips and tension measurement. Preparation of helical strips of endothelium-free rat-tail arterial smooth muscle and tension measurement were as described previously [10]. The strips [0.5 mm × (6–7) mm] were fixed between two hooks of a force displacement transducer system (TB-612T, Nihon Kohden, Tokyo, Japan) at 75 mg resting tension in 750 μl of Hepes–Tyrode (H–T) solution (137 mM NaCl/2.7 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5.6 mM glucose/10 mM Hepes, pH 7.4). After the strips were held at this resting tension in H–T solution at room temperature for 45 min, it was replaced with that containing 1 μM prazosin and 0.1 μM propranolol for 10 min to block the α_1- and β-adrenergic effects of noradrenaline, which is released from nerve terminal by depolarization. Then, the strips were exposed to 60 mM KCl H–T solution for 15 min. Smooth muscle contraction at this step was evoked by Ca^{2+} influx through voltage-gated Ca^{2+} channels. After washing with Ca^{2+}-free H–T solution (H–T solution containing 2 mM EGTA in place of 1.8 mM $CaCl_2$) for 5 min, the strips were stimulated with Ca^{2+}-free H–T solution containing 20 mM caffeine for 5 min to induce contraction by release of Ca^{2+} from the sarcoplasmic reticulum *via* ryanodine receptor. This series of treatments was repeated 3 times and identical contractile responses were observed at each measurement step for the same strip. Next, the muscle strips were incubated with the lamprey protein in H–T solution for 30 min. Then effect of the protein on the contraction was examined first in the KCl H–T solution for 15 min and then in caffeine H–T solution for 5 min as described above. For measuring the contractile force, all these H–T solutions contained the respective concentrations of prazosin and propranolol in addition to the indicated concentrations of the protein. KCl H–T solution (60 mM) was prepared

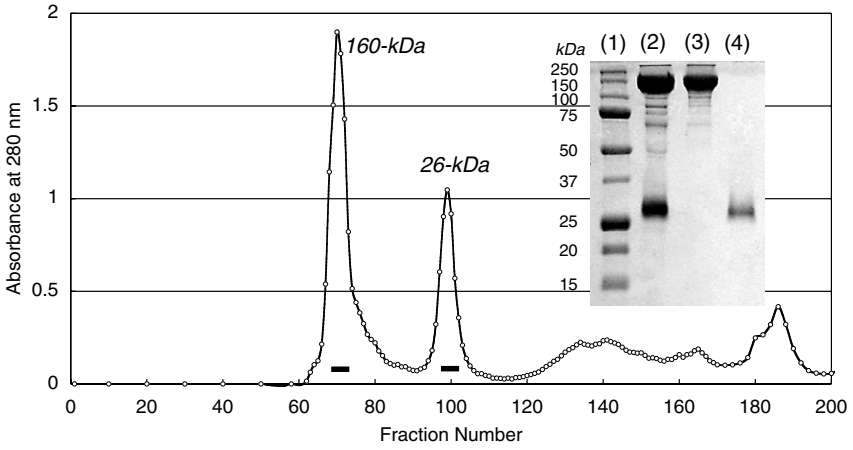

Fig. 1. Gel filtration of the buccal gland secretion on a Sephacryl S-100 column. A column (1.5 × 100 cm) was equilibrated and eluted with 10 mM Tris–HCl/0.1 M NaCl, pH 8. Elution was monitored by absorbance at 280 nm. Fractions (3 ml) were collected and pooled as indicated with horizontal bars. Inset shows SDS–PAGE of the purified proteins on 12% gel. (1) Marker proteins, (2) buccal gland secretion, (3) 160-kDa protein, (4) 26-kDa protein.

by replacing the NaCl in H–T solution with equimolar KCl. All the solutions were pre-oxygenated with 100% O_2.

Results and discussion

Fractionation of the buccal gland secretion

The buccal gland secretion was brown-colored, rather dense solution as previously described for sea lamprey's secretion [11]. Dark brown precipitate was removed by centrifugation and supernatant was separated into several fractions by gel filtration on the Sephacryl S-100 column (Fig. 1). Composition of the secretion was rather simple. There were two major protein peaks designated as 160-kDa protein and 26-kDa protein, from their molecular masses determined by SDS–PAGE. The value for the latter was refined to 25,780 by MALDI-TOF-MS. These results are in consistent with those in the report of Xiao, et al. [8] who also found two major proteins of 159,909 and 25,660 molecular weights determined by mass spectrometry.

Primary structure of 160-kDa protein

Purified 160-kDa protein was reduced and *S*-pyridylethylated and digested with lysyl endopeptidase. Resultant peptides were separated by reversed-phase HPLC and selected four peptides were sequenced. Homology search performed on the UniProt (Universal Protein Resource) sequence database revealed that they were all identical to portions of the amino acid sequence of Japanese river lamprey plasma albumin released recently (UniProt Accession No. A2V8B9) as follows: LAHRFEVLAE (to the residues 274–283 of river lamprey plasma albumin), AAEFHNRV VWQIAHRYPTAG (residues 366–385), FSHSVTEC

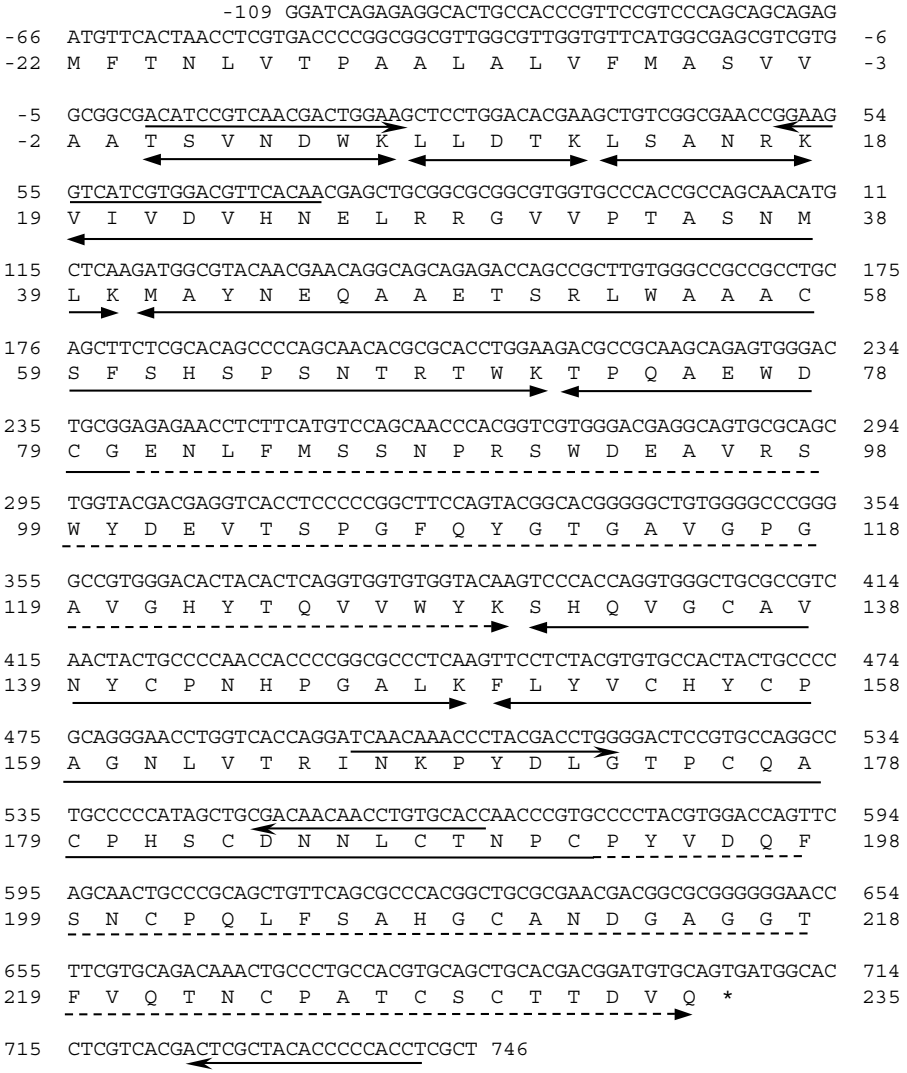

Fig. 2. Nucleotide and amino acid sequences of lamprey 26-kDa protein. Amino acid sequences determined by direct analysis of the lysyl endopeptidase peptides are shown with solid lines. Dashed lines indicate the region confirmed only by amino acid composition. Primers used for amplification and sequencing are indicated under the nucleotide sequence with right (forward) and left (reverse) arrows. Numbering is based on the mature protein. Putative signal sequence is the residues −22 to −1. Asterisk (*) indicates the stop codon.

CAXDK (identical to the residues 1178–1190, except for an unidentified residue X), and SDVSEQGACFK (residues 1300–1310). The amino-terminal sequence was EAEXF (80% identity, X, unidentified residue). The large molecular mass of the protein also supported its identity with lamprey plasma albumins, which comprised of 1394 (sea lamprey [12]) and 1372 (river lamprey) amino acid residues, more than 2-fold that of mammalian plasma albumin. Xiao, et al. [8] reported Ca^{2+}- and Mg^{2+}-dependent fibrinogenolytic activity of a 160-kDa protein in the buccal gland secretion with amino-terminal sequence, EAESFQNLKT RICGGLNGLG. Although they did not mention, this amino-terminal sequence is clearly homologous to that of sea lamprey plasma albumin, EDESFPHLKS RLCGGL NGLG (UniProt **Q91274**, 75% identity) and identical to that of Japanese river lamprey albumin. This means that lamprey plasma albumin is a metalloprotease. They also recognized its cytolytic activity to human carcinoma cell lines. Plasma albumin is usually regarded as a carrier of low molecular mass molecules, such as fatty acids and bilirubin [13]. Interestingly, strong trypsin inhibitor activity was reported for a frog plasma albumin [14] and it seems that plasma albumin can exhibit much more diverse biological activities than previously thought.

Primary structure of 26-kDa protein

The amino-terminal 22-amino acid sequence was determined by direct sequencing of the protein. Eight lysyl endopeptidase peptides of the pyridylethylated protein were separated by reversed-phase HPLC except for one precipitated. The sum of their amino acid compositions accounted for that of the protein (data not shown). These peptides were completely sequenced to the carboxyl-termini except for two large peptides (Fig. 3).

cDNA-cloning of 26-kDa protein

Oligo dT-primed first strand cDNA was prepared from total RNA of the buccal glands. PCR using a pair of degenerated primers set to the amino-terminal TSVNDWK (forward) of the protein and CDNNLCT (reverse) in peptide K-8 could amply cDNA corresponding to amino terminal 189 amino acid residues. This sequence could be extended to the carboxy-terminal end and further 41 nucleotides by using a forward primer, 5′-TC AAC AAA CCC TAC GAC CTG G-3′ (Ile166-Gly173). A reverse sequencing primer was set at this non-coding region and the coding sequence was confirmed on the complementary strand. The amino acid sequence thus determined was in consistence with the results of protein analysis. Sequence of signal peptide region was determined by 5′-RACE against the full-length cDNA library using a gene-specific reverse primer 5′-TTG TGA ACG TCC ACG ATG ACC TTC C-3′ (RKVIVDVHN, residues 17–24). Cleavage site of the putative signal sequence was predicted to be between Ala-Thr (Fig. 2) by the SignalP 3.0 Server [15], which was consistent with the amino-terminal of the purified protein. The com-

Fig. 3. Comparison of lamprey 26-kDa protein and reptile venom CRISP proteins. Lamprey protein is aligned with representative venom CRISP proteins of snakes and lizards. Identical residues are marked with asterisks. Cysteine residues are in shaded blocks. Universal Protein Resource (UniProt) Accession Numbers are as follows. Snakes: Q8UW25; *Lamemis hardwicii* (Hydrophiidae), Q7T1K6; *Naja atra* (natrin-1, Elapidae), Q8JI38; *Laticauda semifasciata* (latisemin, Elapidae), Q8AVA4; *Pseudechis australis* (pseudechetoxin, Elapidae), Q8JI40; *Agkistrodon halys blomhoffi* (ablomin, Viperidae), Q8JI39; *Trimeresurus flavoviridis* (triflin, Viperidae). Lizards: Q9055; *Heloderma horiidum horridum* (helothermine, Helodermatidae), Q2XXP1; *Varanus varius* (VAR-11, Varanidae). Taxonomic names are taken from the database.

plete primary structure of 26-kDa protein is shown in Fig. 2.

Homology search performed by BLAST with the UniProt database revealed that the lamprey protein is a member of cysteine-rich secretory protein superfamily (CRISP) (Fig. 3). CRISP proteins are widely distributed extracellular proteins found in yeasts, plants and animals but their exact function is as yet unknown [16,17]. These proteins contain strictly conserved 16 cysteine residues and are consisted of an amino-terminal pathogenesis-related protein-like domain and a carboxy-terminal cysteine-rich domain separated by a short peptide segment [17]. As shown in Fig. 3, five insertions of 2–5 amino acids are present in the lamprey CRISP when aligned with venom CRISP proteins, which may be due to the large evolutionary distance between lampreys and reptiles, and might have brought some functional difference to the lamprey protein. Since many reptile venom CRISP proteins block various ion channels, they may be regarded as neurotoxins. With this in mind, we examined the effect of lamprey CRISP protein on the ion channel activity.

The effect of lamprey CRISP (26-kDa protein) on rat-tail arterial smooth muscle contraction

As shown in Fig. 4, lamprey CRISP protein inhibited high K^+-induced contraction of rat-tail arterial smooth muscle. Strength of the inhibition was concentration-dependent to 1 μM of the lamprey protein, then somewhat reduced at a higher concentration (3 μM). The inhibition was completely reversible and high K^+-induced contraction was fully restored upon washout of the protein (data not shown). These were similar to those observed for snake venom CRISP proteins [16]. Contraction of smooth muscle induced by high K^+ treatment is a result of membrane-depolarization, subsequent activation of voltage-gated Ca^{2+}-channels and resultant Ca^{2+} influx. Therefore, inhibition of the arterial smooth muscle contraction by lamprey CRISP can be regarded as blockage of voltage-gated Ca^{2+}-channels, especially L-type Ca^{2+}-channels among the channel subtypes, since major Ca^{2+}-channels in rat-tail smooth muscle cells are L-type Ca^{2+}-channels [18]. One of the toxic effects of venom CRISP proteins is blockage of ryanodine receptors observed for the lizard

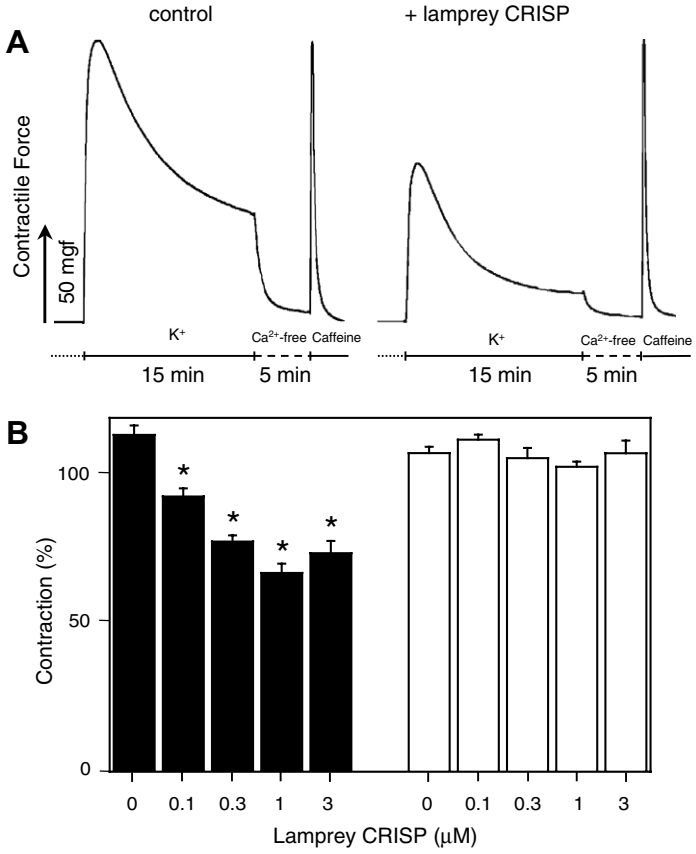

Fig. 4. Effect of lamprey 26-kDa protein (CRISP) on the contraction of rat-tail arterial smooth muscle. (A) A typical trace showing the inhibition of high K^+-induced contraction by lamprey CRISP. Smooth muscle strips were pretreated with 1 μM CRISP, then exposed to stimulants (60 mM K^+ or 20 mM caffeine). For experimental details, see Materials and methods. (B) Concentration dependency of the inhibition of high K^+ (left) or caffeine (right) stimulated contraction by lamprey CRISP ($n = 6$, mean ± SEM, $^*P < 0.001$). Contractile force induced by the stimulants in the Hepes–Tyrode solution was taken as 100%. Slight increase of the force was observed for both stimulants when Tris–HCl, in which lamprey CRISP was dissolved, was present during the measurement (B, 0 μM CRISP).

toxin, helothermine [19]. Fig. 4 shows that there was no effect of lamprey CRISP on the contraction of the arterial smooth muscle induced by caffeine-treatment, which will activate ryanodine receptors of the sarcoplasmic reticulum.

Obviously snake and lizard had evolved their venom toxins to exert potentially lethal effects on the preys and predators. Lampreys, however, attach to larger fishes and feed mainly on blood. In the case of sea lampreys the duration of a single feeding attachment to host fishes was rather long, from 76 to 220 h depending on the body size of the lampreys [20]. During that period host fishes should remain alive, although fishes may eventually die from blood loss. Therefore, lamprey CRISP in the buccal gland secretion, unlike toxins in snake and lizard venom, needs not be lethal. Its function may be to prevent contraction of the local blood vessels and to keep the site of attachment bleeding in cooperation with lamphredin anticoagulants. The blood-sucking hemipteran insect, *Rhodnius prolixus*, is also a slow feeder, and secretes vasodilating heme protein nitrophorins (also called prolixin S) from salivary glands. Nitrophorins store nitric oxide and gradually release it into the host tissues. Released nitric oxide binds to the soluble guanylate cyclase, leading to smooth muscle relaxation and vasodilatation through activation of a signaling cascade [21]. Besides direct inhibition of coagulation cascade, hematophagous animals have evolved a number of strategies to facilitate blood sucking, such as inhibition of platelet aggregation, reduction of inflammation, and induction of vasodilatation [7,21]. Lamprey CRISP seems to be another example of mechanisms adapted to feeding on vertebrate blood. In addition, blockage of Ca^{2+} channels might have some other local and/or systemic effects on the host fish advantageous to the parasitism. A recent transcriptome analysis on venom toxins of lizards and snakes revealed that CRISP and several other toxins are common to lizards and snakes, indicating their very ancient origin dating back to about 200 million years ago [22]. These reptile venom gland CRISP proteins could be a direct descendant of lamprey buccal gland CRISP.

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