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

Naoko Ito [a], Mitsuo Mita [b], Yoshiaki Takahashi [c], Ayano Matsushima [a,d], Yuichi G. Watanabe [a,d], Shigeki Hirano [c], Shoji Odani [a,d,*]

[a] *Life Science Course, Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan*
[b] *Department of Pharmacodynamics, Meiji Pharmaceutical University, Tokyo 204-8588, Japan*
[c] *School of Health Science, Faculty of Medicine, Niigata University, Niigata 951-8518, Japan*
[d] *Department of Biology, Faculty of Science, Niigata University, Niigata 950-2181, Japan*

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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

* Corresponding author. Address: Department of Biology, Faculty of Science, Niigata University, Niigata 950-2181, Japan. Fax: +81 25 262 6116.
E-mail address: sodani@bio.sc.niigata-u.ac.jp (S. Odani).

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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

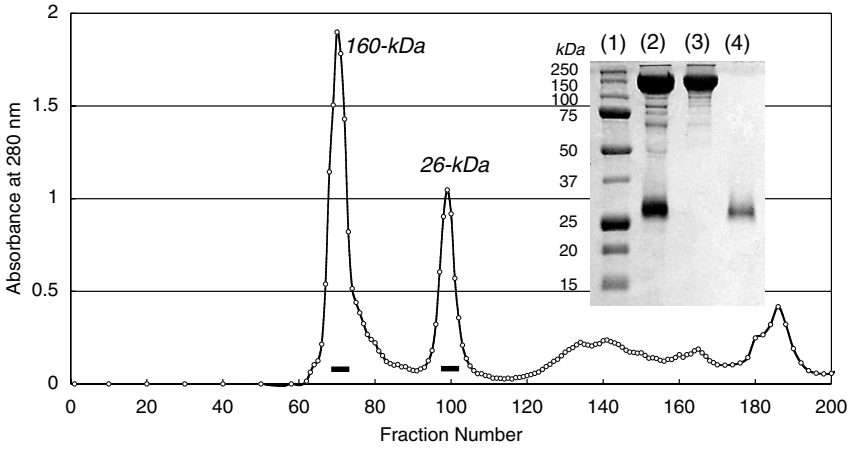

Fig. 1. Gel filtration of the buccal gland secretion on a Sephacryl S-100 column. A column (1.5 × 100 cm) was equilibrated and eluted with 10 mM Tris–HCl/0.1 M NaCl, pH 8. Elution was monitored by absorbance at 280 nm. Fractions (3 ml) were collected and pooled as indicated with horizontal bars. Inset 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.