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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Hirudins of the Asian medicinal leech, *Hirudinaria manillensis*: same same, but different

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Abstract

Blood coagulation in vertebrates is a complex mechanism that involves the precisely coordinated and regulated action of a cascade of factors in order to prevent excessive blood loss upon wounding. Any blood sucking ectoparasite, however, has to circumvent this mechanism to ensure the uptake of an adequate blood meal. Inhibitors of blood coagulation in the saliva are hence widespread among these animals. Thrombin as a key factor of blood coagulation is a prominent target of such inhibitors, and hirudin is probably the best known among the thrombin inhibitors. Hirudin was originally described in the genus *Hirudo*, but occurs in other leech genera like *Hirudinaria* and *Macrobdella* as well. Besides several isoforms of hirudin, a new class of putative leech saliva components, the hirudin-like factors (HLFs), was identified in both genera *Hirudo* and *Hirudinaria*. Here, we describe the expression, purification, and functional characterization of three HLFs (HLF5, 6, and 8, respectively) and two additional hirudins (HM3 and HM4) of *Hirudinaria manillensis*. While HLF6 lacked any inhibitory activity on thrombin, HLF5 as well as HLF8 clearly exhibited anticoagulatory properties. The inhibitory activity of HLF5 and HLF8, however, was much lower compared with both HM3 and HM4 of *Hirudinaria manillensis* as well as the hirudin variants 1 (HV1) and 2 (HV2) of *Hirudo medicinalis*. Neither an inhibition of trypsin nor a platelet aggregation was caused by HLF8. Our data indicates the presence of two classes (rather than isoforms) of hirudins in *Hirudinaria manillensis* with markedly different inhibitory activity on human thrombin.

Keywords Hirudin · Hirudin-like factors · *Hirudinaria manillensis* · Blood coagulation · Medicinal leeches

Introduction

In the pre-genomic era, identification and characterization of proteins with properties of interest usually followed the principle “function seeks factor.” At first, there often was an observation and the description of an apparent (biological) effect, e.g., the prolonged bleeding following a leech bite or the almost complete inhibition of coagulation of blood that was mixed with leech head extracts. Subsequently, biological material like tissue samples, culture supernatants, or leech saliva were processed and fractionated using a set of preparative and analytical methods (like HPLC) and appropriate activity assays. At its best, finally, only the pure protein of interest remained to be further analyzed. Using this approach, Fritz Markwardt was able to isolate and characterize the thrombin inhibitor hirudin (Markwardt 1955, 1957; Markwardt 1967). With pure hirudin in hand, it was possible to determine its primary structure (Petersen et al. 1976; Bagdy et al. 1976; Dodt et al. 1984, 1986). Only later, the respective cDNAs (Harvey et al. 1986) and genes (Scacheri et al. 1993; Müller et al. 2016) of different hirudins were identified.

The genomic era almost completely reversed the scenario. Proteome analysis and mass spectrometry allowed the identification of yet unknown peptides and proteins, and transcriptome analysis by screening of cDNA libraries enabled the determination of their respective cDNAs. Whole genome sequencing projects even provide access to the data of thousands of putative genes encoding already known and yet unknown (hypothetical) proteins. “Factor seeks function” is the

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direct consequence, and this turns out to be a difficult and very challenging task.

The saliva of hematophagous leeches may serve as a good example to illustrate the problem. It is a complex mixture of proteins and small bioactive molecules (Baskova and Zavalova 2001; Baskova et al. 2008; Hildebrandt and Lemke 2011). Once secreted into the host during a blood meal, the bouquet of factors may display a broad spectrum of activities ranging from prolonged bleeding over pain relief to inhibition of bacterial growth (Hildebrandt and Lemke 2011; Sig et al. 2017). However, only a few of these factors have already been identified and characterized in detail (Rigbi et al. 1996; Salzet 2001; Hildebrandt and Lemke 2011), but many more are predicted to occur (Baskova et al. 2004; Yanes et al. 2005). For several leech species, transcriptome data of salivary glands are available (Min et al. 2010; Macagno et al. 2010; Kvist et al. 2014; Hibsh et al. 2015; Siddall et al. 2016; Tessler et al. 2018; Lu et al. 2018; Khan et al. 2019). At best, these data sets cover the whole spectrum of leech saliva protein factors and hence may contain all the information necessary to identify the yet unknown components. But the problem is the one mentioned above: how to link a predicted protein derived from a cDNA (or even genomic) sequence to a biological function?

The host spectrum of haematophagous leeches comprises representatives of all groups of vertebrates, but even conspecies may serve as targets of attack (Sawyer 1986; Wilkin and Scofield 1990; Keim 1993; Merilä and Sterner 2002; Lai and Chen 2010). Both the successful uptake of a sufficient amount of blood and its long-term storage essentially depend on the efficient prevention of blood coagulation by the leech. The blood coagulation system in vertebrates is highly conserved, and hemostasis comprises two interconnected steps: platelet plug formation (or primary hemostasis) and clot formation (or secondary hemostasis). Whereas the former one is affected by platelet aggregation inhibitors, the latter one is affected by inhibition of factor Xa (e.g., by antistasin) or thrombin (e.g., by hirudin). Thrombin already occurs in jawless fish (lamprey) (Doolittle 2009), and thrombin molecules of different vertebrates (human, duck, clawed frog, zebra fish, shark, lamprey) share 60–70% identical and 75–85% similar amino acid residues including almost fully conserved residues determining exosite 1, exosite 2, and the catalytic center (Lane et al. 2005; Ponczek et al. 2012; personal observations). Hirudin is a bivalent inhibitor of thrombin, blocking the access of fibrinogen to the catalytic center of thrombin (through the N-terminal amino acid residues) on the one hand and on the other hand the fibrinogen binding site (trough the acidic C-terminal tail) (Chang 1983; Stone and Hofsteenge 1986; Rydel et al. 1991; Warkentin 2004). Both modes of action can be experimentally separated (Wallace et al. 1989; Chang et al. 1990; DiMaio et al. 1990; Schmitz et al. 1991). Decorsins of *Macrobdella decora* (Say, 1824) (Seymour et al. 1990), ornatins of *Placobdella ornata* (Verrill, 1872) (Mazur et al. 1991), LAPP of *Haementeria officinalis* (Blanchard, 1896) (Connolly et al. 1992; Keller et al. 1992), calin (Munro et al. 1991) and saratin (Barnes et al. 2001) of *Hirudo medicinalis* are inhibitors of platelet aggregation. The molecular structure of calin is still unknown. Saratin and LAPP share a high degree of sequence and structural homology in core regions (Gronwald et al. 2008), but are both very different from hirudins. Decorsin and ornatin, however, possess typical structural features of hirudins and HLFs (e.g., six conserved cysteins) yet contain an additional RGD (or KGD) motif between cysteine residues 5 and 6 that is essential to bind to integrin αIIbβ3 (GP IIb/IIIa) (Ruoslahti and Pierschbacher 1987; Krezel et al. 2000).

A class of putative leech saliva proteins of yet unknown function are the hirudin-like factors (HLFs). HLFs share typical structural features of hirudin, e.g., six conserved cysteine residues that, by forming disulfide bridges, stabilize the N-terminal core unit (Corral-Rodríguez et al. 2010; Koh and Kini 2011). Nonetheless, they lack other features like the canonical PKP and DFxxIP motifs that are proposed to be essential for hirudin's ability to block thrombin's proteolytic activity or for binding to fibrinogen (Dodt et al. 1988; Rydel et al. 1990; Grütter et al. 1990). So far, HLFs have been identified in the genera *Hirudo* (HLF1-4) and *Hirudinaria* (HLF5, 6, and 8) (Müller et al. 2016; Müller et al. 2017). Of these, only HLF1 has been successfully expressed, purified, and functionally tested in a coagulation assay, where it did not show any signs of anticoagulatory activity (Müller et al. 2016). The aim of the present study was to evaluate the potential biological activities of the HLFs of *Hirudinaria manillensis* (Lesson, 1842). In addition, the predicted anticoagulatory activity of HM3 and HM4, two putative hirudins of *Hirudinaria manillensis* (Müller et al. 2017), remained to be verified.

Material and methods

Genotyping of animals and tissue preparation

The biological material used in this study (specimen of *Hirudinaria manillensis* and salivary gland preparations) was already described by Müller et al. (2017). Information on genotyping data and GenBank accession numbers can be obtained from the same publication.

Overexpression and purification of His-tagged HLFs and hirudins

The procedure to clone cDNAs encoding HLFs and hirudins, to overexpress and purify the respective proteins, was previously described in detail (Müller et al. 2016). Briefly, we applied a system developed by Qiagen (Hilden, Germany). The pQE30Xa vector encodes a factor Xa protease recognition site between the His-tag coding region on the 5′ side and the multiple cloning site on the 3′ side. Factor Xa protease treatment cleaves off the His-tag and results in a recombinant protein that is free of any vector-derived amino acids at the N-terminus. Partial cDNAs of HLFs, HM3, and HM4 were cloned into pQE-30Xa in a way that the first amino acid of the respective hirudin or HLF (without the signal sequence) was located directly adjacent to the factor Xa protease cleavage site. The cDNA sequences of interest were amplified using the primer pairs listed in Table 1 and Phusion™ High-Fidelity DNA polymerase (Thermo Scientific, Schwerte, Germany). For the overexpression, pQE30Xa-clones containing inserts encoding the hirudin or HLF variants of interest were transformed into appropriate *Escherichia coli* strains. Two flasks, each containing 500 ml of LB medium with ampicillin, were inoculated with 10 ml of a preculture. From the start of inoculation, optical densities were determined in a regular frequence. At an $OD_{600} = 0.5$, the expression of hirudin and HLF variants was induced by adding IPTG to a final concentration of 1 mmol/l. After 4 h of expression, cells were harvested, the pellet was carefully resuspended in binding buffer (20 mmol/l Tris/HCl, 500 mmol/l NaCl, 5 mmol/l imidazole, pH 7.9), and the cells were sonicated using a Sonopuls homogenizer (Bandelin, Berlin, Germany). After centrifugation for 1 h at 4 °C and 4500 rpm (approx. 3900*g*) in a Labofuge 400R (Thermo Scientific, Schwerte, Germany), the supernatant was carefully removed, filtered through a filter with a pore size of 0.45 μm, and loaded on a self-packed column containing Ni–IDA His-Bind® resin (Merck, Darmstadt, Germany). Washing and elution steps were performed as recommended by the manufacturer of the resin. Equal volumes of every fraction were analyzed by SDS-PAGE on 20% gels. Prior to the treatment with factor Xa protease, fractions of interest were dialyzed twice for 24 h at 4 °C against a 100-fold excess of reaction buffer (20 mmol/l Tris/HCl, 100 mmol/l NaCl, 2 mmol/l $CaCl_2$, pH 8.0). Volume reduction by using Spectra/Gel™ Absorbent (SGA) (Roth, Karlsruhe, Germany) was performed to increase protein concentration. All steps were carried out in a dialysis membrane with a MWCO of 5000 (Roth, Karlsruhe, Germany). The final volume was approximately 2 ml.

Table 1 List of oligonucleotide primers used in the study

Primers used for cloning of HM3 into expression vector pQE30Xa	
Forward	5′-GTG AGC TAC ACT GAT TGT ACG GAG-3′
Reverse	5′-CAT AAG CTT ATT ATT TCA TGT CAA TGT CAC-3′
Primers used for cloning of HM4 into expression vector pQE30Xa	
Forward	5′-GTG AGT TAC AGC GGA TGT ACG-3′
Reverse	5′-GAA AGC TTC GTA ACT TAT ATG AG-3′
Primers used for cloning of HLF5 into expression vector pQE30Xa	
Forward	5′-ATG GTT TAC ACA GAA TGT TC-3′
Reverse	5′-GCA ATT GGA AGC TTT TTT CAC-3′
Primers used for cloning of HLF6 into expression vector pQE30Xa	
Forward	5′-GTA CGT TTC ATG AAA TGT TCG G-3′
Reverse	5′-GGT TTA AGC TTA ATT CTA ATC G-3′
Primers used for cloning of HLF8 into expression vector pQE30Xa	
Forward	5′-TTA ACT TAT AGA GAC TGT TCG C-3′
Reverse	5′-TTA AGC TTC GTT TAT CTT CGA TAG TCA TC-3′

Factor Xa protease treatment and purification

The treatment of fusion proteins containing the factor Xa protease recognition sequence consisted of three steps: (1) factor Xa protease cleavage, (2) removal of factor Xa protease, and (3) cleanup of the digested protein. All steps were performed as recommended by the manufacturer (Qiagen, Hilden, Germany). Purity of recombinant hirudins and HLFs was confirmed by SDS-PAGE on 20% gels. Molar concentrations of protein solutions were calculated by dividing the absorbance at 280 nm by the molar absorption coefficient according to the equation $\varepsilon = (nW \times 5500) + (nY \times 1490) + (nC \times 125)$ (Gill and von Hippel 1989; Pace et al. 1995).

Blood coagulation assays

To verify the biological activity of purified HLFs and hirudins, three blood coagulation assays were performed: the activated partial thromboplastin time test (aPTT; reference range 22.7–28.9 s), the prothrombin time test (PT; reference range 10.7–13.7 s), and the thrombin time test (TT; reference range 16.8–21.4 s) using a BFT II analyzer (Siemens Healthcare, Erlangen, Germany). All steps followed the instructions outlined by the manufacturer. For the coagulation assays, all protein samples were diluted with dialysis buffer to reach final concentrations of 75 μmol/l, 7.5 μmol/l, and 0.75 μmol/l, respectively. The desired amount of substrate was transferred directly into the cuvette immediately before the plasma was added. Dade® Ci-Trol® 1 (Siemens Healthcare, Erlangen, Germany) was used as standardized human plasma. The incubation of reaction mixtures was carried out at 37.4 °C. Measurements that lasted up to 300 s were stopped and declared as a complete inhibition of clot formation.

Generation of duckling plasma

For a comparative analysis of the inhibitory effect of hirudin or HLF variants on thrombins of different origins, we developed a coagulation assay based on duck plasma instead of human plasma. Blood of ducklings of *Anas platyrhynchos* at age 10–11 days was retrieved and collected in a falcon tube (Sarstedt, Nürnbrecht, Germany) prefilled with 1 ml of a 109 mmol/l sodium citrate buffer (85.5 mmol/l tri-sodium citrat dihydrate, 23.4 mmol/l citric acid monohydrate, pH =

5.6) until a final volume of 10 ml was reached. Falcon tubes were immediately inverted four times and centrifuged at 2500*g* for 15 min at 15 °C. Supernatant was transferred into a fresh falcon tube and centrifuged again at 2500*g* for 15 min at 15 °C. Finally, aliquots of 1 ml were generated, immediately frozen in liquid nitrogen, and stored at − 80 °C.

Platelet aggregation assay

The study was performed with human blood samples and approved by the institutional ethics committee. After written informed consent was obtained from healthy volunteers, 10 ml of venous blood were taken from the antecubital vein using a S-Monovette® (Sarstedt, Nürnbrecht, Germany) prefilled with citrate buffer. The blood sample was separated in 1 ml aliquots with cutoff pipette tips to reduce shear forces and kept at 37 °C. A first centrifugation step was performed at 1000 rpm (approx. 100*g*) for 5 min. After the centrifugation, samples were allowed to sediment further for 5 min at 37 °C, then the supernatant (platelet-rich plasma, PRP) was transferred into clean Eppendorf tubes for further processing. The remaining blood was centrifuged again for 4 min at 6000 rpm (approx. 4000*g*). The supernatant was dedicated as platelet-poor plasma (PPP), transferred into new micro reaction vessels, and used as a reference value for maximal platelet aggregation.

Platelet aggregation was measured using an APACT-4004 (LABiTec, Ahrensburg, Germany). The potent platelet aggregation inhibitors tirofiban and eptifibatide (Sigma-Aldrich, Taufkirchen, Germany) were used for control purposes. Volume of 10 μl of the respective test and control compounds (75 μmol/l) or dialysis buffer was transferred into cuvettes and 180 μl of PRP were added, giving a final sample concentration of 3.8 μmol/l. The cuvettes were transferred into the APACT-4004 measuring cell and the measurement was started. After 1 min, 10 μl of a collagen solution (100 μg/ml; Hart Biologicals, Hartlepool, UK) were added as an agonist. All experiments were performed at 37 °C over a time period of 7 min. Maximal aggregation in percentage and maximal slope of the curve in percentage per minute were calculated as quantitative output parameters (Zhou and Schmaier 2005).

Trypsin activity assay

A slightly modified method after Erlanger et al. (1961) was used to determine an inhibitory effect of hirudins or HLFs on trypsin activity. Volumes were adjusted to carry out the assays in transparent 96 well plates (Sartedt, Nürnbrecht, Germany). One microliter of 75 μmol/l stock solutions of each sample or 1.6 μl of a 0.1 mg/ml soybean trypsin inhibitor solution (Applichem, Darmstadt, Germany) were transferred into wells and adjusted to a volume of 23.4 μl with *Aqua Bidest*. 9.6 μl of a standard trypsin solution used for cell culture purposes (2.5 g/l, PAN-Biotech, Aidenbach, Germany) was added. After a pre-incubation of 5 min, 167 μl of the substrate solution (1 mmol/l Nα-benzoyl-L-arginine-4-nitroanilide hydrochloride (BAPA) in 0.01% dimethyl sulfoxide (DMSO), 50 mmol/l Tris-HCl, 20 mmol/l $CaCl_2$, pH = 8.2 were added to an end volume of 200 μl. The absorbance at a wavelength of $\lambda = 410$ nm was measured every 30 s over a period of 5 min in a standard plate reader (Tecan, Crailsheim, Germany). The calculation of the specific trypsin activity (in U/mg) was done with the following equation (adapted from Sørensen et al. 1999):

$$\mathrm{U/mg} = (\mathrm{A_{410}/min{\cdot}GV{\cdot}VF})/(\varepsilon{\cdot}d{\cdot}\mathrm{PV{\cdot}mg/ml})$$

(A_{410}/min is the alteration of the extinction at $\lambda =$ 410 nm min, GV is the whole sample volume in ml, VF is the dilution factor, ε is the molar attenuation coefficient in square centimeters per micromole, d is the thickness of the cuvette in centimeters, PV is the amount of trypsin solution in milliliters and milligrams per milliliter is the concentration of trypsin solution)

Results

The main aim of this study was to evaluate the putative anticoagulatory activity of HLF5, 6, and 8 as well as to verify the respective activity of hirudins HM3 and HM4. These are all factors that are derived from the Asian medicinal leech, *Hirudinaria manillensis*. Two hirudin variants of its European counterpart *Hirudo medicinalis* (Linnaeus, 1758), hirudin-VV (hirudin variant 1, HV1) and hirudin-IT (hirudin variant 2, HV2), served as references. All factors were overexpressed, purified, and processed as described above in the "Material and Methods" section.

HM3 and HM4 are full hirudins

In the coagulation tests of activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT), 500 pmol of HM3 and HM4, and HV1 (as a reference) were used in a final reaction volume of 156.7 μl. No clot formation was detectable over a measurement period of 5 min (Fig. 1), indicating full inhibition of blood coagulation by all factors. Consequently, both HM3 and HM4 can be considered full hirudins.

HLF5 and HLF8 are unorthodox hirudins

To evaluate putative anticoagulatory activities of HLF5, HLF6, and HLF8, we performed the aPTT and the TT tests. The latter one is the most sensitive method to detect a direct

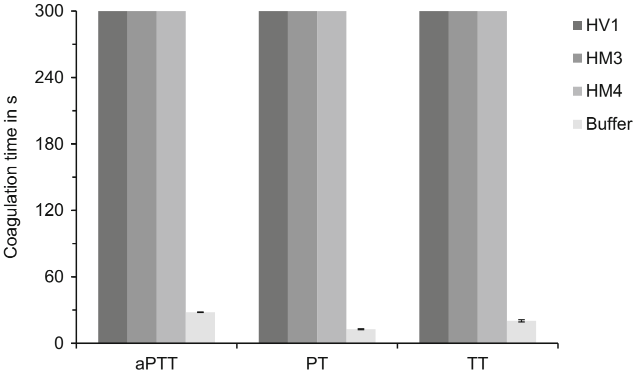


Fig. 1 Standard blood coagulation assays with hirudin variants HV1 (VV) of *Hirudo medicinalis*, HM3 and HM4 of *Hirudinaria manillensis*. aPTT, activated partial thromboplastin time test; PT, prothrombin time test; TT, thrombin time test. Concentration of factors was 3.2 µmol/l. Results are mean of three independent measurements

inhibitory activity on thrombin (Walenga et al. 1991). Again, HV1 served as a positive control. To our surprise, both HLF5 and HLF8 clearly showed anticoagulatory effects by inhibiting thrombin (Fig. 2). However, the inhibitory activities of HLF5 and HLF8 in both tests were markedly lower than the one of HV1. In contrast, HLF6 did not show any effect in either of the tests. Consequently, HLF5 and HLF8 must be considered as hirudins and not as hirudin-like factors. However, to emphasize the apparent differences between "hirudins" like the hirudin-variants HV1-3 of *Hirudo medicinalis* and HM1-4 of *Hirudinaria manillensis* on the one hand and "hirudin-like factors comprising antithrombotic activity" like HLF5 and 8 on the other hand, we propose to use from now on the terms "full hirudins" and "unorthodox hirudins," respectively.

Duck plasma as a coagulation test component

As already mentioned in the "Introduction," thrombins of different vertebrates share a high degree of sequence similarity and identity. However, only a few substitutions might already be sufficient to cause changes in affinity of different thrombins to different hirudin variants and thus may even enhance the anticoagulatory effect of unorthodox hirudins. For this reason, we developed coagulation assays based on duck plasma instead of human plasma (as it is used in standard assays) and tested the hirudin variants HV1 and HV2 as well as HLF8 upon their activity on duck thrombin. All factors prolonged the coagulation time in both the aPTT and the PT tests when compared with buffer as a negative control (Fig. 3). But again, the effect of HLF8 was much less pronounced, supporting its classification as an unorthodox hirudin.

Effect of an N-terminal His-tag

Hirudin is a bivalent inhibitor of thrombin, blocking the access of fibrinogen to the catalytic center of thrombin (through the three N-terminal amino acid residues) on the one hand and on the other hand the fibrinogen binding site (trough the acidic C-terminal tail) (Chang 1983; Stone and Hofsteenge 1986; Rydel et al. 1991; Warkentin 2004). Both modes of action can be experimentally separated (Wallace et al. 1989; Chang et al. 1990; DiMaio et al. 1990; Schmitz et al. 1991). The His-tag encoded by pQE30Xa comprises 21 amino acids with the sequence "MRGSHHHHHHGSGSGSGIEGR." Due to its size and charge, a His-tag at the N-terminus of hirudin blocks any interaction with the catalytic site of thrombin with certainty, but most likely leaves the C-terminal tail of hirudin unaffected. Full hirudins of *Hirudo medicinalis* and *Hirudinaria manillensis* show a high degree of amino acid sequence identity or similarity among all parts of the molecule. In contrast, especially the C-terminal tails of the unorthodox hirudins HLF5 and 8 differ markedly in length and sequence (see Fig. 4). By testing N-terminal His-tag variants of HV1 and HV2 as well as HLF8, we tried to evaluate whether the interaction between thrombin and the unorthodox hirudins follows the same bivalent mode as described for the full hirudins or relies mainly on blocking of the catalytic site of thrombin alone. Our data indicate that an N-terminal His-tag indeed decreases the anticoagulatory activity of HV1, HV2, and

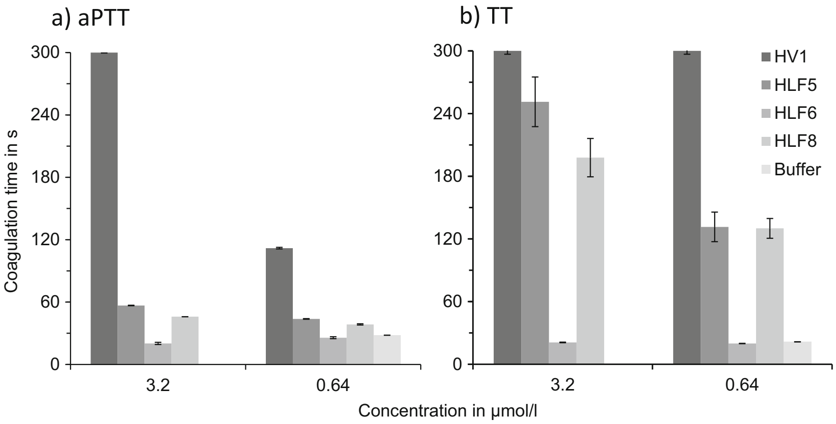


Fig. 2 Standard blood coagulation assays with hirudin variant HV1 (VV) of *Hirudo medicinalis* and HLF variants HLF5, 6, and 8 of *Hirudinaria manillensis*. **a** Activated partial thromboplastin time test (aPTT). **b** Thrombin time test (TT). Results are mean of three independent measurements

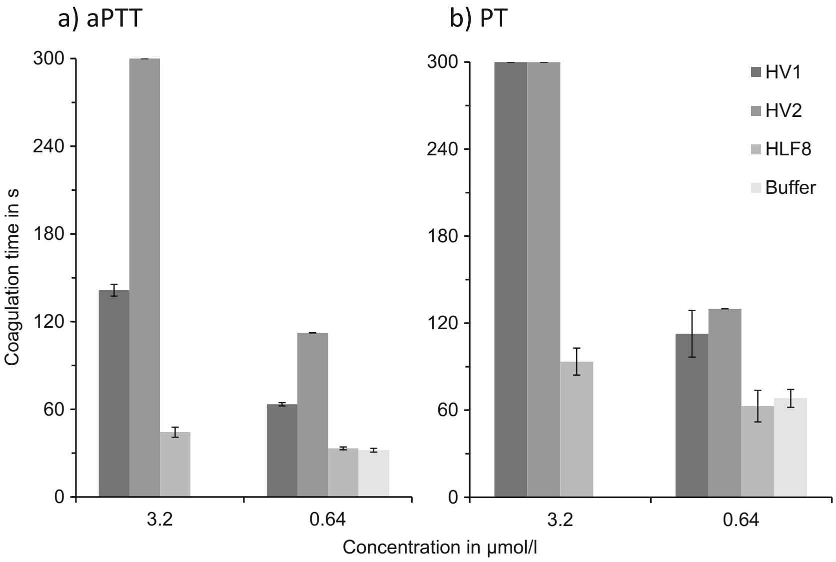


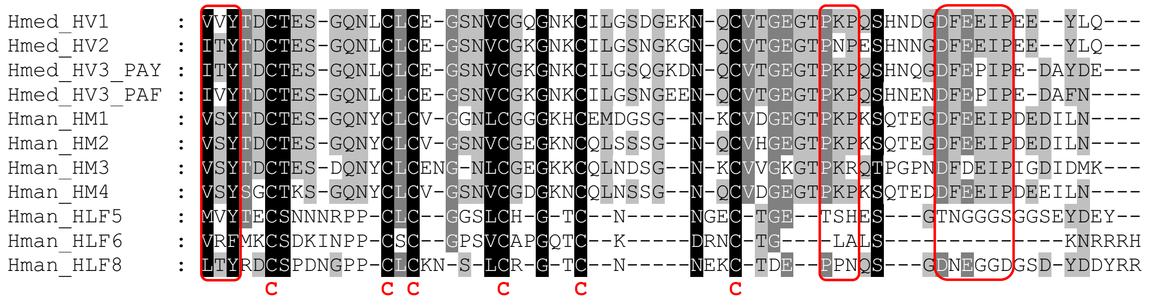
Fig. 3 Blood coagulation assays using duck plasma with hirudin variants HV1 (VV) and HV2 (IT) of *Hirudo medicinalis* (HV1) and HLF8 of *Hirudinaria manillensis*. **a** Activated partial thromboplastin time test (aPTT). **b** Prothrombin time test (PT). Results are mean of three independent measurements

HLF8 dramatically (Fig. 5). However, His-HV1 and His-HV2 retained some activity at least at high concentrations (3.2 µmol/l), whereas there was almost no detectable anticoagulatory activity of His-HLF8. Hence, HLF8 (and probably also HLF5) are most likely monovalent inhibitors of thrombin.

Platelet aggregation and trypsin activity are not affected by HLF8

Decorsin and ornatin are inhibitors of platelet aggregation but possess typical structural features of hirudins and HLFs (e.g., six conserved cysteins). In addition, they contain an RGD (or KGD) motif located between cysteine residues 5 and 6 that is essential to bind to integrin αIIbβ3 (GP IIb/IIIa) (Ruoslahti and Pierschbacher 1987; Krezel et al. 2000). HLF8 does not contain an RGD motif at a corresponding position, but SGD and GGD motifs are located within the C-terminal tail. HLF5 does not contain any of these motifs. For this reason, we only tested HLF8 for its putative inhibitory effect on platelet aggregation. HV1 and HV2 served as negative controls (meaning, no inhibition of platelet aggregation is expected; Glusa and Markwardt 1990); tirofiban and eptifibatide were used in equimolar amounts as positive controls (both substances are potent inhibitors of platelet aggregation used in clinical practice; Nurden et al. 1999). As can be seen in Fig. 6a–c, neither the hirudin variants HV1 and HV2 nor HLF8 showed any significant negative effect on platelet aggregation, while both tirofiban and eptifibatide had a clear inhibitory activity.

Several inhibitors of trypsin activity have been isolated from leeches, including bdellastasin (Moser et al. 1998) and hirustasin (Söllner et al. 1994) of *Hirudo medicinalis* and bdellin-HM (Lai et al. 2016) of *Hirudinaria manillensis*. Putative trypsin-inhibitory effects of hirudin variants HV1 and HV2 and of HLF5, 6, and 8 were tested in a chromatographic assay. Neither of the factors had any negative effect on trypsin activity (Fig. 7).



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Hmed_HV1     : VVYTDCTES-GQNLCLCE-GSNVCGQGNKCILGSDGEKN-QCVTGEGTPKPQSHNDGDFEEIPEE--YLQ---
Hmed_HV2     : ITYTDCTES-GQNLCLCE-GSNVCGKGNKCILGSNGKGN-QCVTGEGTPNPESHNNGDFEEIPEE--YLQ---
Hmed_HV3_PAY : ITYTDCTES-GQNLCLCE-GSNVCGKGNKCILGSQGKDN-QCVTGEGTPKPQSHNQGDFEPIPE-DAYDE---
Hmed_HV3_PAF : IVYTDCTES-GQNLCLCE-GSNVCGKGNKCILGSNGEEN-QCVTGEGTPKPQSHNENDFEPIPE-DAFN----
Hman_HM1     : VSYTDCTES-GQNYCLCV-GGNLCGGGKHCEMDGSG--N-KCVDGEGTPKPKSQTEGDFEEIPDEDILN----
Hman_HM2     : VSYTDCTES-GQNYCLCV-GSNVCGEGKNCQLSSSG--N-QCVHGEGTPKPKSQTEGDFEEIPDEDILN----
Hman_HM3     : VSYTDCTES-DQNYCLCENG-NLCGEGKKCQLNDSG--N-KCVVGKGTPKRQTPGPNDFDEIPIGDIDMK---
Hman_HM4     : VSYSGCTKS-GQNYCLCV-GSNVCGDGKNCQLNSSG--N-QCVDGEGTPKPKSQTEDDFEEIPDEEILN----
Hman_HLF5    : MVYTECSNNNRPP-CLC--GGSLCH-G-TC--N-----NGEC-TGE--TSHES---GTNGGGSGGSEYDEY--
Hman_HLF6    : VRFMKCSDKINPP-CSC--GPSVCAPGQTC--K-----DRNC-TG----LALS---------------KNRRRH
Hman_HLF8    : LTYRDCSPDNGPP-CLCKN-S-LCR-G-TC--N-----NEKC-TDE--PPNQS---GDNEGGDGSD-YDDYRR
                    C      C C      C     C                C
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Fig. 4 Multiple sequence alignment of hirudin and HLF variants. Comparison of hirudin variants HV1-3 of *Hirudo medicinalis*, HM1-4 of *Hirudinaria manillensis* and HLF variants HLF5, 6, and 8 of *Hirudinaria manillensis*. The alignments were generated using ClustaX2 (Larkin et al. 2007) and manually edited. Black background indicates conserved residues; gray background indicates similar residues. The six conserved cysteine residues giving rise to the three-dimensional structure are marked in bold. The conserved PKP and DFxxIP motifs of hirudins are boxed. Abbreviations are used according to the IUPAC code

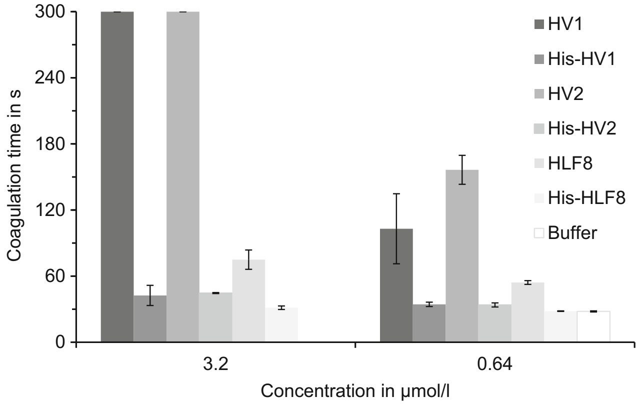

Fig. 5 Standard blood coagulation assays activated partial thromboplastin time test (aPTT) with hirudin variants HV1 and HV2 (IT) of *Hirudo medicinalis* and HLF8 of *Hirudinaria manillensis* with and without the N-terminal His-tag. Results are mean of three independent measurements

Discussion

Medicinal leeches are fascinating creatures from many different points of view. As zoologists, we are impressed by their unique Bauplan and physiological capabilities. For a physician, they are valuable assistants for the treatment of human maladies. An artist is possibly inspired by their beautiful body coloration and elegant style of swimming. In contrast, the interest of a natural product chemist is most likely focused on their intrinsic values: dozens or even a hundred of bioactive factors contained in the saliva and transferred into the host during feeding. Most of them still remain to be discovered. In the present paper, we have investigated five of such factors originating from the Asian medicinal leech, *Hirudinaria manillensis*. Our approach was to "seek functions for factors," as outlined in the "Introduction."

Two of these factors, HM3 and HM4, were predicted to be hirudins (Müller et al. 2017). Predictions were solely made based on typical structural features common to all hirudins, like the canonical PKP and DFxxIP motifs that are essential for hirudin's ability to block thrombin's proteolytic activity or for binding to fibrinogen (Dodt et al. 1988; Rydel et al. 1990; Grütter et al. 1990). Our coagulation assay data strongly supports the original assumptions (Fig. 1). HM3 and HM4 both exhibited inhibition of blood coagulation comparable to the

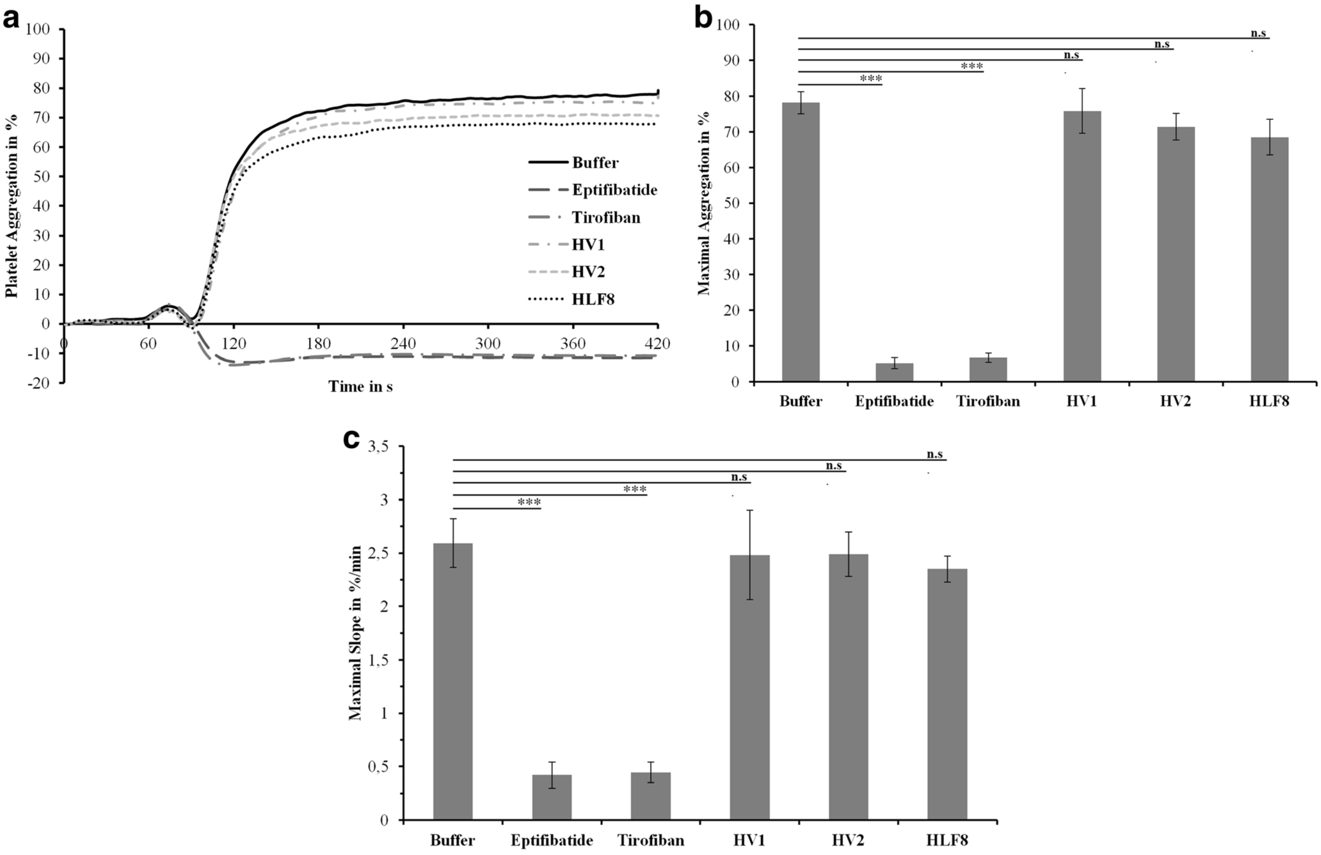

Fig. 6 Collagen-induced platelet aggregation assays with hirudin variants HV1 (VV) and HV2 (IT) of *Hirudo medicinalis* and HLF8 of *Hirudinaria manillensis*. Tirofiban and eptifibatide were used as positive controls for complete inhibition of platelet aggregation, buffer as a negative control. Results are mean of four independent measurements. **a** Platelet aggregation curves. **b** Values of maximal aggregation. **c** Values of maximal slope. Asterisks indicate significant differences ($p < 0.001$). n.s. not significant

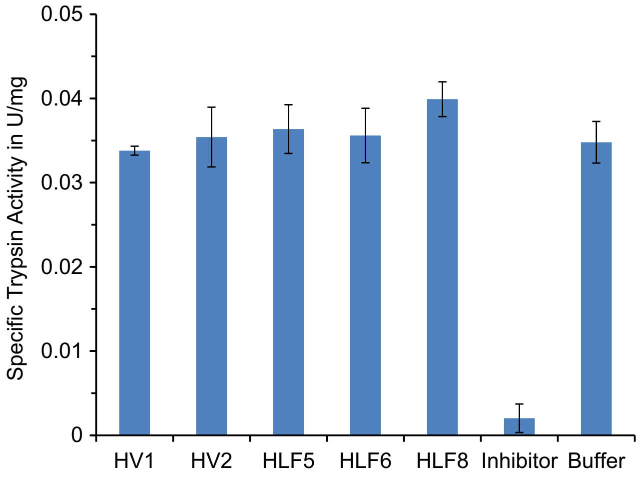

Fig. 7 Trypsin activity assays with hirudin variants HV1 (VV) and HV2 (IT) of *Hirudo medicinalis* and HLF variants HLF5, 6, and 8 of *Hirudinaria manillensis*. Soybean trypsin inhibitor was used as positive control for complete inhibition of trypsin activity, buffer as a negative control. Results are mean of four independent measurements

hirudin variants HV1 and HV2 of *Hirudo medicinalis* despite differences in the host preferences of both leech species. Whereas *Hirudo medicinalis* preferentially feeds on amphibians and water fowl, but rarely on mammals; the main hosts of *Hirudinaria manillensis* are amphibians, reptiles, and mammals, but rarely, if at all, birds (Sawyer 1986; Wilkin and Scofield 1990; Keim 1993; Merilä and Sterner 2002; Lai and Chen 2010).

The hirudin-like factors are a class of leech saliva components that share typical structural features of hirudins, e.g., six conserved cysteine residues and a common gene structure composed of four exons and three introns (Müller et al. 2016), but lack other features like the motifs mentioned above (Fig. 4). Nevertheless, to our surprise, both HLF5 and HLF8 turned out to be inhibitors of thrombin and hence the blood coagulation cascade. The inhibitory effect, however, was markedly lower compared with the hirudin variant HV1 of *Hirudo medicinalis* (Fig. 2). HLF6, in contrast, did not show any sign of thrombin inhibition (Fig. 2). A comparison of the primary structure of the three HLFs of *Hirudinaria manillensis* (Fig. 4) did not reveal obvious evidence for the difference, but the isolectric points (pI values; pH value of a solution at which the net electrical charge of a protein becomes zero) considerably differ. The isoelectric points of HLF5 and HLF8 are 4.30 and 4.27, respectively. These values are perfectly within the range of pI values calculated for hirudins (about 4.06 for HM4 and 4.47 for HM3, only as an example). For HLF6, the isoelectric point is about 9.61. A different occurrence of basic versus acidic amino acid residues is evident in particular within the C-terminal tail of the HLFs. Additionally, the tail of HLF6 is much shorter (Fig. 4). For hirudins, the C-terminal tail confers binding to the exosite I of thrombin, the fibrinogen binding site, by electrostatic interactions (Betz et al. 1991; Sharp 1996). A shorter tail and hence a disturbed sterical capability for such interactions may explain the reduced inhibitory activity of HLF5 and 8 toward thrombin. Hirudin HM2 fragments 1-41 and 1-47, generated by the deletion of the C-terminal 17 or 23 amino acid residues of HM2 of *Hirudinaria manillensis*, are competitive inhibitors of thrombin. However, the binding to thrombin is much less efficient than that of intact hirudin HM2. Furthermore, the fragment 1-47 is a better inhibitor than fragment 1-41 (Vindigni et al. 1994). The very short and highly basic tail of HLF6 very likely does not interact with exosite I of thrombin, but this is not a conclusive explanation for the complete lack of antithrombotic activity of HLF6. Interaction between the N-terminus of HLF6 and the catalytic site of hirudin must also be hindered too. It is possible that this is due to the presence of the two basic amino acid residues Arg2 and Lys5 (Fig. 4).

The question, whether HLF5 and 8 are mono- or bivalent inhibitors of thrombin, remains open. Our remarks above point to a bivalent mode of action that involves both the N-terminus and the C-terminal tail. In contrast, the data on N-terminal His-tagged variants of HV1, HV2, and HLF8 tell a different story. An N-terminal His-tag certainly completely blocks the insertion of hirudin's N-terminal residues into the catalytic site of thrombin, altering it to a monovalent inhibitor. Consequently, both His-tagged HV1 and HV2 are much less effective, but retained some anticoagulatory activities (Fig. 5) that can be explained by the binding of the C-terminal tail to exosite I of thrombin. In contrast, the activity of His-tagged HLF8 almost completely faded. We therefore assume that HLF8 (and probably also HLF5) is rather a mono- than bivalent inhibitor, targeting the catalytic site of thrombin through their N-terminal residues. The C-terminal tail may only have a supportive and stabilizing function. Only co-crystallographic analyses of HLFs and thrombin can help to solve these open problems.

HLF5 and HLF8 are probably representatives of a new class of thrombin inhibitors, and we propose the term "unorthodox hirudins" for this class. Functionally, they are located between full hirudins like HV1 and HV2 of *Hirudo medicinalis* or HM3 and HM4 of *Hirudinaria manillensis* and the hirudin-like factors without any thrombin-inhibitory activity. So far, no representative of unorthodox hirudins has been described in members of the genus *Hirudo*. However, the hirudin of the North American medicinal leech, *Macrobdella decora*, may qualify for the yet exclusive club. Very recent studies indicate a very similar activity profile of hirudin$_{\mathrm{Mdec}}$ compared with HLF5 and 8 (Müller et al. 2019). Remarkably, hirudin$_{\mathrm{Mdec}}$ seems to be the only hirudin variant expressed in *Macrobdella decora* (Min et al. 2010). *Hirudinaria manillensis*, in contrast, simultaneously expresses both classes of hirudins. This observation gives rise to the question about the biological significance of the unorthodox hirudins particularly. We propose two alternative explanations.

First, the real targets of unorthodox hirudins are different from thrombin, and thrombin inhibition is only an evolutionary relict. By incorporation of a RGD motif between cysteine residues 5 and 6 of hirudin variant HV1, Mo and colleagues constructed a chimeric hirudin molecule comprising of both anti-thrombin and anti-platelet aggregation activities (Mo et al. 2009; Huang et al. 2014). Hence, one activity does not necessarily prevents the other. So far, we have tested the HLFs of *Hirudinaria manillensis* in two additional functional assays: efficiency of platelet aggregation and trypsin activity. No negative effect of either of the factors was observed. More tests are mandatory, and at least for HLF6, the biological target remains elusive: factor still seeks function.

Second, full and unorthodox hirudins may act differentially on different thrombins and/or at different ambient temperatures. As already mentioned above, thrombins of different vertebrate taxa display a very high degree of overall sequence similarity/identity (Lane et al. 2005; Ponczek et al. 2012; personal observations). However, they are not completely identical, and they originate from organisms with markedly different body temperatures. Fish and amphibians are poikilothermal organisms; their body and blood temperature corresponds to the surrounding temperature and may be far below 20 °C when they get attacked by a leech. In waterfowl, a counter current exchange system reduces blood temperature in feet and thereby heat loss in naked or poorly insulated extremities (Midtgård 1980; Midtgård 1981). Feet of waterfowl are certainly the preferred sites of leech attacks. Taken together, leeches may take up blood of very diver origins and temperatures and hence may require a set of hirudins with different biochemical properties to ensure inhibition of blood coagulation under all conditions. In contrast, standard coagulation assays are performed with human plasma and at the human body temperature of about 37 °C. Strictly speaking, data of such experiments are of limited value. In a first attempt, we have developed and performed additional coagulation assays using duck plasma as a key component. Both full hirudins and HLF8 markedly prolonged the coagulation time, but the effect of HLF8 was again much less pronounced. For now, there is no indication that the factors tested actually act differentially on human and duck thrombin. Coagulation tests with plasma of amphibians or fish, performed at lower temperatures, may be reasonable projects for the future.

Taken together, we have only partially solved the problem of assigning functions to factors, namely yet uncharacterized hirudins and hirudin-like factors of the Asian medicinal leech, *Hirudinaria manillensis*. The predicted hirudins, HM3 and HM4, are indeed fully active on thrombin, comparable to hirudins of the European medicinal leech, *Hirudo medicinalis*. Of the hirudin-like factors, HLF5 and 8 surprisingly turned out to be hirudins as well, but form a new class of, to our definition, unorthodox hirudins. For HLF6, however, we still seek its function.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval We declare that the experiments described in this paper comply with the current laws in Germany. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures in our studies involving human participants (platelet aggregation experiments) were approved by the local ethics committee and performed according to the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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