

KOMPONENTE MODRASOVEGA STRUPA Z VPLIVOM NA SRČNO-ŽILNI SISTEM

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SRČNO-ŽILNI SISTEM

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COMPONENTS OF THE NOSE-HORNED VIPER VENOM THAT
AFFECT CARDIOVASCULAR SYSTEM

Doctoral Dissertation

Mentor: prof. dr. Igor Križaj

Somentorica: doc. dr. Adrijana Leonardi

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Posvećeno mojemu očetu Mladenu Latinoviću.

Počivaj v miru!

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Povzetek

Bolezni srca in ožilja so po najnovejšem poročilu Svetovne zdravstvene organizacije vodilni vzrok smrti v svetu (31 %). Trenutne terapije žal povzročajo tudi resne stranske učinke, zato potekajo intenzivne raziskave na področju razvijanja novih, in predvsem bolj varnih zdravil za zdravljenje kardiovaskularnih bolezni. Strupi kač iz družine Viperidae so posebno bogati s proteini, ki vplivajo na proces strjevanja krvi. Z namenom odkrivanja naravnih učinkovin, primernih za razvoj novih in varnih zdravil za terapijo kardiovaskularnih bolezni ali za hematološko diagnostiko, smo temeljito raziskali strupa dveh lokalnih viperid, modrasa in gada, najbolj strupene in pa najbolj razširjene evropske kače.

Primerjava proteomov modrasovega in gadovega strupa nam je podala molekularno razlago razlike v toksičnosti med obema strupoma. Modrasov strup je precej bolj kompleksen od gadovega. Vsebuje večji nabor toksičnih komponent, pa tudi višje koncentracije metaloproteinaz P-III, ki povzročajo močno krvavitev. Oba strupa se izrazito razlikujeta po vplivu na strjevanje krvi. Medtem, ko je modrasov strup izrazito antikoagulanten, je gadov prokoagulanten. Med drugim je ta razlika posledica vsebnosti antikoagulantnih amoditoksinov, sekretornih fosfolipaz A₂, izključno v modrasovem strupu. Amoditoksini so sicer tudi nevrotoksični. Zato so nevrotoksični učinki v primeru zastrupitve z modrasovim strupom pogosti, v primeru zastrupitve z gadovim pa izjemno redki. Zaradi večjega nabora hemostatsko aktivnih komponent v modrasovem strupu smo tega uporabili kot izhodišče za njihovo analizo. Posebno pozornost smo posvetili komponentam z antikoagulantnim delovanjem. Razvili smo kompleksne kromatografske postopke za izolacijo homogenih in farmakološko aktivnih molekul iz strupa. Učinek izoliranih proteinov na koagulacijo krvi smo določali s standardnimi kliničnimi testi na komercialno dostopni plazmi. Detajle molekularnih mehanizmov delovanja izoliranih strupnih komponent na strjevanje krvi, smo določali z uporabo čistih faktorjev strjevanja krvi. Opisali smo metaloproteinazo VaF1, ki specifično cepi α -verigo fibrinogena. Sodi v družino metaloproteinaz P-IIIa. Za razliko od sorodnih metaloproteinaz VaF1 praktično ni hemoragična, kar ji daje precejšnje možnosti v smeri razvoja sredstva za defibrinogenacijo krvi. Odkrili in opisali smo protein VaaSPH-1, ki zavira koagulacije krvi po notranji poti. Protein strukturno sodi med serinske proteaze, je pa encimsko neaktiven. Ugotovili smo, da je antikoagulantna aktivnost VaaSPH-1 posledica vezave te molekule na aktivirani koagulacijski faktor VIII (FVIIIa), kar prepreči vezavo FIXa in nastanek intrinzičnega tenaznega kompleksa, s tem pa strjevanje krvi po notranji poti. Določili smo del strukture VaaSPH-1, ki tvori kontakt s FVIIIa, in tako podali možnost za zasnovo varnih antikoagulantov za zdravljenje venske tromboembolije, ki jih medicina močno pogreša. Nadalje smo opisali močno prokoagulantno serinsko proteazo VaaSP-VX in pojasnili tudi mehanizem njenega delovanja. Deluje tako, da verjetno proteolitsko aktivira FV in FX, ki potem tvorita protrombinazni kompleks, potreben za koagulacijo krvi. Molekula je zelo primerna za razvoj lokalnega zdravila proti hemofiliji, saj spodbuja nastanek krvnega strdka na mestu poškodbe. Zaradi načina delovanja, bi ta molekula lahko tudi nadomestila manj zanesljivi reagent, razredčen strup Russellovega gada, pri testiranju lupusnih antikoagulantov. Z namenom izboljšanja terapije zastrupitev smo se posvetili tudi slabo raziskani kardiotoksičnosti modrasovega in gadovega strupa. Kot glavno komponento z delovanjem na srce smo v obeh strupih identificirali amoditin L, encimsko neaktivno sekretorno fosfolipazo A₂.

Abstract

Cardiovascular diseases are the leading cause of death worldwide (31%) according to the latest World Health Organization report. Intensive research is being conducted to obtain new drugs, as current therapies cause serious side effects. Venoms from the snake family Viperidae are particularly rich in pharmacologically interesting proteins, which mainly influence the blood clotting process. In an attempt to discover new natural components suitable for the further development of safer drugs for the treatment of cardiovascular diseases or for haematological diagnostics, we studied toxins from two local vipers, nose-horned viper, *Vipera ammodytes ammodytes* (*Vaa*), and the common viper or adder, *Vipera berus berus* (*Vbb*), the most poisonous and the most common European snake, respectively.

The proteomic comparison of the *Vaa* and *Vbb* venoms has provided us with an explanation at the molecular level for the differences in the toxicity of these two venoms. The first and most important distinction is that the *Vaa* venom contains more diverse toxic components. We have also observed higher concentrations of P-III class metalloproteinases in *Vaa* venom, which are the cause of more severe bleeding in humans upon envenomation by *Vaa*. There is also a noticeable difference in the activity of both venoms, namely *Vbb* venom has a procoagulant effect, while *Vaa* venom has an anticoagulant effect. Among molecules responsible for the anticoagulant effect of the *Vaa* venom are amodytoxins, which we have identified only in this venom. Amodytoxins are also responsible for neurotoxic effects, which have been rarely observed in the case of *Vbb* venom poisoning, probably because they are not present in this venom. Because of the larger pool of haemostatically active components in *Vaa* venom, we have used this venom for further studies. Special attention was paid to the isolation of anticoagulant components. Therefore, we have developed complex chromatographic methods to purify haemostatically active molecules from the venom. The effect of the isolated proteins on blood coagulation was tested using standard clinical tests on commercially available plasmas. Based on the effect that the isolated toxins had on the homogeneous coagulation factors, we also determined their molecular mechanism of action. We discovered a metalloproteinase with fibrinogenolytic activity, VaF1, which specifically cleaves an α -chain of fibrinogen. As such, it has therapeutic potential as a defibrinogenating agent. It belongs to the P-IIIa class of metalloproteinases, which, in contrast to related enzymes, has no significant haemorrhagic activity. We have also identified and characterized a protein that inhibits the intrinsic pathway of blood coagulation, *Vaa*SPH-1, which belongs to the family of serine proteases but is devoid of enzymatic activity. The anticoagulant activity of *Vaa*SPH-1 is a consequence of its binding to FVIIIa antagonizing in this way the binding of a natural partner molecule FIXa. The identified structural features of *Vaa*SPH-1 that are responsible for its interaction with FVIIIa provided an excellent basis for the development of safe medically useful anticoagulants, which are greatly missed in the treatment of venous thromboembolism. Simultaneously with the isolation of *Vaa*SPH-1, another serine protease was isolated, *Vaa*SP-VX. The approach described above helped us to define its mechanism of action, *i.e.* activation of FV and FX. *Vaa*SP-VX is a suitable substance for the development of a new drug to promote blood clot formation. Due to its potentially dual, FV and FX, activation activity, *Vaa*SP-VX is also interesting for lupus anticoagulant testing to replace the unreliable diluted Russell's viper venom. Additionally, our intention was to improve the current therapy after *Vaa* and *Vbb* poisoning by identifying cardiotoxins in their venoms. We identified ammodytin L, an enzymatically inactive myotoxic secreted phospholipase A₂, as the most important cardiotoxin in both venoms.

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Kratice

Aminokislina	Tričrkovna oznaka	Enočrkovna oznaka
alanin	Ala	A
arginin	Arg	R
asparagin	Asn	N
asparaginska kislina	Asp	D
cistein	Cys	C
fenilalanin	Phe	F
glicin	Gly	G
glutamin	Gln	Q
glutaminska kislina	Glu	E
histidin	His	H
izolevcin	Ile	I
levcin	Leu	L
lizin	Lys	K
metionin	Met	M
prolin	Pro	P
serin	Ser	S
tirozin	Tyr	Y
treonin	Thr	T
triptofan	Trp	W
valin	Val	V

α 2-AP	...	α 2-antiplazmin
α 2-MG	...	α 2-makroglobulin
ADAM	...	družina proteinov, ki vsebujejo domeno, podobno disintegrinom, in domeno, podobno metaloproteinazam
ADP	...	adenozin difosfat
anti- <i>Vaa</i>	...	protistrup, proizveden proti celotnemu modrasovemu strupu
anti-H	...	protistrup, proizveden proti hemoragični frakciji modrasovega strupa
Atn	...	amoditin
Atx	...	amoditoksin
Ca^{2+}	...	kalcijev ion
cDNA	...	komplementarna deoksiribonukleinska kislina
CRISP	...	sekretorni proteini, bogati s cisteinom
devLVP	...	razviti tlak levega prekata
dLVP	...	levi diastolični ventrikularni tlak
domena C	...	domena bogata s cisteinom
domena D	...	domena, podobna disintegrinom
EKG	...	elektrokardiogram
FX (FV, FVII, ...)	...	koagulacijski faktor X (V, VII ...)
FXa (FVa, FVIIa, ...)	...	aktivirani koagulacijski faktor X (V, VII ...)
kDa	...	kilodalton
KS	...	kačji strup
LAEO	...	L-aminokislinske oksidaze
MP	...	metaloproteinaza
MS	...	masna spektrometrija
NaDS-PAGE	...	poliakrilamidna gelska elektroforeza v prisotnosti natrijevega dodecilsulfata
NOAC	...	novi peroralni antikoagulant
PAI-1	...	zaviralec tkivnega aktivatorja plazminogena
pI	...	izoelektrična točka
PLTC	...	proteini, podobni lektinom tipa C
RP-HPLC	...	visokotlačna tekočinska kromatografija visoke ločljivosti z obrnjenima fazama
serpin	...	proteinski zaviralec serinskih proteaz
sLVP	...	levi sistolični ventrikularni tlak
SP	...	serinska proteaza
sPLA ₂	...	sekretorna fosfolipaza A ₂
TFPI	...	zaviralec poti tkivnega faktorja
TLE	...	trombinu podobni encimi
t-PA	...	tkivni aktivator plazminogena
TXA ₂	...	tromboksan A ₂
<i>Vaa</i>	...	<i>Vipera ammodytes ammodytes</i>
<i>Vbb</i>	...	<i>Vipera berus berus</i>
vWF	...	von Willebrandov faktor
u-PA	...	urokinaza
Zn^{2+}	...	cinkov ion
2DE	...	dvodimenzionalna poliakrilamidna gelska elektroforeza

Poglavje 1

Uvod

1.1 Kačji strupi

Kače so mesojedi plazilci, ki jih uvrščamo v red luskarjev. Njihovi sorodniki so želve, krokodili in kuščarji. Prehranjujejo se z majhnimi živalmi, kot so kuščarice, druge kače, mali sesalci, ptiči, in njihovimi jajci, ribami, polži ali žuželkami. Večina kač ni strupenih in ubijejo plen, tako da ga pogoltnejo ali zadušijo s stiskanjem. Za razliko od njih strupene kače uporabljajo strup, kompleksen koktajl peptidov in beljakovin, kot smrtonosno orožje za lov in obrambo. Strup proizvajajo in hranijo v strupnih žlezah, ob ugrizu pa ga vbrizgajo v žrtev skozi ostre strupnike ali ob njih. Strup prizadene vitalne telesne funkcije žrtve ali napadalca, na primer povzroči mišično ohromelost, prepreči dihanje, vpliva na proces strjevanja krvi itd. [1].

Od približno 3000 do sedaj opisanih kač je petina strupenih. Te so razvrščene v štiri družine: *Colubridae* (goži), *Elapidae* (strupeni goži), *Atractaspidae* (zemeljski gadi) in *Viperidae* (gadi) [1]. Družina kač *Viperidae* se naprej deli na poddružine: *Azemiopinae*, *Crotalinae* (jamičarke), *Causine* (nočni gadi) in *Viperinae* (pravi gadi) [2]. V slednjo sodi tudi šest evropskih vrst kač, in sicer: *Vipera latastei* (Latastejev gad), *Vipera xanthina* (otomanski gad), *Vipera ursinii* (mali gad), *Vipera aspis* (laški gad), *Vipera berus* (navadni gad) in *Vipera ammodytes* (modras). Zadnje tri živijo tudi na področju Slovenije in njihov strup ima precejšen medicinski pomen.

Slovenske strupenjače prepoznamo po čokatem telesu, zelo kratkem repu in ozkih pokončnih zenicah, pogosto pa se jim po hrbtu vije tudi značilen cikcakast vzorec. Ta vzorec je pri navadnem in laškem gadu včasih lahko nepovezan in tvori prečne proge. Pri obeh vrstah pogosto srečamo tudi popolnoma črne primerke brez vzorca. Navadni gad lahko zraste v dolžino tudi do 90 cm, v povprečju pa doseže okoli 70 cm (slika 1.1 – desno). Modras je prepoznaven po značilnem rožičku na nosu in temnem cikcakastem vzorcu na svetlejšem hrbtu (slika 1.1 – levo). V dolžino meri od 65 do 95 cm. Naseljuje skalnata, suha in prisojna področja južne Evrope [3].

Ocenjuje se, da je povprečno število zdravstvenih posredovanj zaradi zastrupitve po ugrizu kače med letoma 1970 in 2010 v Evropi znašalo 7992 na leto (kar je približno 1 ugriz na 100.000 prebivalcev na leto). Od tega je bilo približno 15 % težjih primerov zastrupitve, 4 % pacientov pa je zaradi posledic zastrupitve po strupenem kačjem ugrizu umrlo [4].



Slika 1.1: Modras (*V. ammodytes ammodytes*) (levo) in navadni gad (*V. berus berus*) (desno). Modrasa prepoznamo po rožičku na nosu (vir: Shutterstock, 2020).

1.1.1 Sestava in učinki kačjih strupov na človeka

Kačji strup (KS) je kompleksna mešanica organskih in anorganskih molekul in ionov [5]. Sestava strupa variira glede na vrsto kače, pa tudi od osebkov do osebkov znotraj iste vrste glede na spol, starost in vrsto prehrane [6, 7]. KS v 90 – 95% sestavljajo organske molekule:

- **polipeptidi brez encimske aktivnosti**, to so na primer proteini, ki delujejo specifično na živčni sistem in celične membrane. V to skupino sodijo proteini, podobni lektinom tipa C (PLTC), disintegrini, triprstni toksini in s cisteinom bogati sekretorni proteini (CRISP). Te molekule inhibirajo proteaze ali delujejo sinergistično z drugimi netoksičnimi komponentami strupa ter peptidi, katerih aktivnost še ni znana;
- **encimi**, ki imajo dve zelo pomembni vlogi, sodelujejo pri usmrtnosti plena in pomagajo pri njegovi prebavi. Mednje sodijo sekretorne fosfolipaze A_2 (sPLA $_2$) serinske proteaze (SP), metaloproteinaze (MP), fosfomono(di)esteraze, 5'-nukleotidaze, hialuronidaze, NAD-nukleotidaze in oksidaze *L*-aminokislin (LAAO).

V KS so prisotni še nukleotidi, biogeni amini ter v zelo majhnih količinah tudi lipidi in aminokisline. Ogljikovi hidrati so prisotni v glavnem v obliki glikoproteinov.

Anorganske sestavine KS so kovinski ioni: Ca^{2+} , Cu^{2+} , Fe^{3+} , K^+ , Mg^{2+} , Mn^{2+} , Na^+ , Co^{2+} in Zn^{2+} . Vsi naštetni ioni niso prisotni v vseh strupih in njihova količina med vrstami variira. Prav tako ni znana biološka vloga vseh, vendar nekateri, na primer Ca^{2+} in Zn^{2+} , igrajo pomembno vlogo pri stabilizaciji strukture proteinov in pri aktivnosti določenih encimov [1].

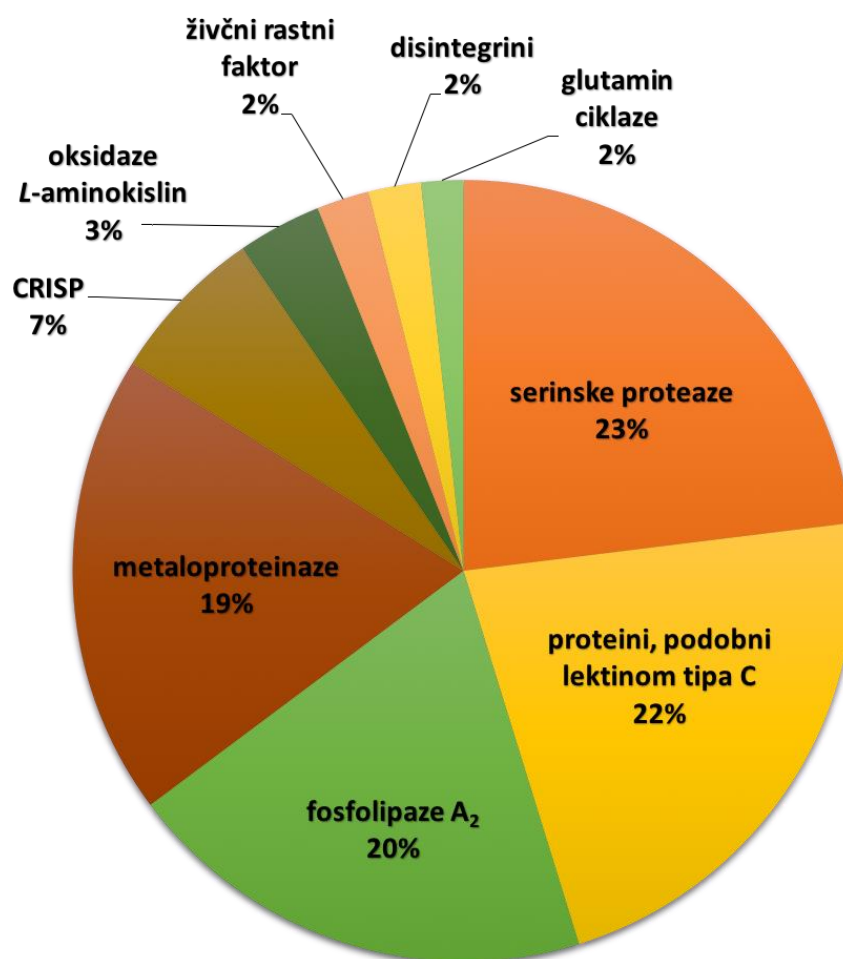
Sestava strupa je specifična za vsako kačo družino. Strupi kač iz družine *Elapidae* delujejo predvsem nevrotoksično in kardiotoksično na človeka, strupi kač iz družine *Atractaspididae* predvsem kardiotoksično, medtem ko so strupi kač iz ostalih dveh družin usmerjeni predvsem na sistem strjevanja krvi – hemostazo. Na splošno strupi viperid poleg motenj strjevanja krvi povzročajo tudi lokalne poškodbe tkiva (zatekanje, edem in nekrozo tkiva) ter znižanje krvnega tlaka [8, 9].

1.1.1.1 Učinki in sestava modrasovega strupa

Modras je najbolj strupena kača v Evropi, ob ugrizu katere so še posebej izraženi naslednji učinki: hemoragija (krvavitev), nekroza tkiva in nevrotoksičnost [10, 11, 12]. Številne komponente iz njegovega strupa delujejo pro- ali antikoagulantno. Delujejo na dva načina: a) s proteolitično aktivnostjo aktivirajo ali pa razgrajujejo komponente koagulacijskega sistema, b) na ne-encimski način, z vezavo na fiziološke tarče preprečijo vezavo fizioloških komponent koagulacijskega sistema in s tem njihovo delovanje [13, 14].

Na mestu ugriza modrasov strup pri človeku povzroči hemoragijo, edem in nekrozo tkiva [10]. Ti učinki so posledica delovanja proteolitičnih encimov v strupu – hemoraginov. V strupu so prisotne tudi nevrotoksične komponente, ki vodijo v delno ali popolno inaktivacijo perifernih živcev (možganskih ali motoričnih), kar se odrazi kot na primer ptoza, oftalmoplegija in ošibitev dihalnega mišičja.

Modrasov strup ima predvsem izrazit vpliv na srčno-žilni sistem človeka [12], za kar so odgovorni proteini iz različnih proteinskih družin: SP, MP, sPLA₂, PLTC ali disintegrinov (slika 1.2) [15]. Zato je modrasov strup zelo primeren vir zdravilnih učinkovin ali pa spojin vodnic za razvoj zdravil za zdravljenje bolezni krvi, srca in ožilja.



Slika 1.2: Zastopanost različnih proteinskih družin v strupu modrasa. Povzeto po [15].

1.1.1.2 Učinki gadovega strupa

Gad je najbolj razširjena kača v Evropi, zato je tudi primerov zastrupitve človeka s strupom te kače največ [4, 16]. Gadov strup v človeku povzroči moteno delovanje hemostatskega sistema, vendar ne tako izrazito, kot ga povzroči modrasov strup. Tudi nevrotoksični učinki so zelo redki. Zastrupitev z gadovim strupom povzroči predvsem zatekanje v okolici ugriza, lahko tudi širše, ter anemijo, ki je posledica krvavitve in hemolize. Pogosto se pojavijo tudi bruhanje, diareja in abdominalne bolečine [10, 17, 18, 19, 20]. Klinična poročila o zastrupitvah z gadovim strupom včasih navajajo tudi toksične učinke na srce [21]. Tako so poročali o pojavu elektrokardiografske inverzije pri pacientih, sploščenju T vala in zvišanju ST intervala (motnje v repolarizaciji membranskega potenciala), atrioventrikularnem bloku druge stopnje (motnje v prevajanju impulzov skozi atrioventrikularni vozle), bradi/tahiaritmiji, atrijski fibrilaciji (motnja srčnega utripa, za katero je značilen nereden in hiter utrip srca) in srčni kapi. Pojav kardiotoksičnosti je, sicer redkeje, opisan tudi pri pacientih po zastrupitvi z modrasovim strupom [22, 23].

Sestava gadovega strupa do sedaj še ni bila dobro poznana, zato je bil naš namen celostno identificirati molekule, ki so prisotne v tem strupu. V poglavju 3.1 je podrobno predstavljena proteomska sestava gadovega strupa, ki smo jo izvedli v okviru doktorskega dela.

1.1.2 Zdravljenje zastrupitev s kačjimi strupi

Razlike med posledicami toksičnih ugrizov se ne pojavljajo le med posameznimi vrstami kač (interspecifično), temveč tudi med posameznimi osebki znotraj iste vrste (intraspecifično). Razlike v posledicah so seveda pogojene z razlikami v sestavi strupov. Znotrajvrstne ali intraspecifične razlike v sestavi strupa so odvisne predvsem od spola živali, starosti in vrste prehrane, ki jo določa predvsem kraj naselitve [6]. V primeru modrasa so na primer analizirali strupe osebkov, ki poseljujejo tri različna področja na Hrvaškem v radiju 150 km, okolico Krapine, Karlovca in Slunja. Pokazali so, da je doza strupa, pri kateri umre polovica mišk (LD_{50}), v primeru strupa modrasov iz okolice Karlovca in Slunja višja kot v primeru strupa modrasov iz okolice Krapine [7].

Pri zdravljenju zastrupitev z modrasovim ali gadovim strupom se uporabljajo protistrupi, ki jih pridobijo bodisi z imunizacijo živali z modrasovim ali gadovim strupom bodisi z mešanico obeh. Na tak način pripravljeni protistrupi so se pokazali učinkoviti tudi pri paraspecifični nevtralizaciji, to je pri nevtralizaciji bolj ali manj podobnih strupov, kot je strup, ki je bil uporabljen za pridobitev protistrupa. Na primer pri nevtralizaciji strupa laškega gada (*Vipera aspis* – Italija) in otomanskega gada (*Vipera xanthina* – Grčija, Turčija). Poleg tega so pokazali učinkovito paraspecifično nevtralizacijo strupov kač iz gorskih predelov Armenije, Raddejevega gada (*Montivipera raddei*) in levantinskega gada (*Macrovipera lebetina*), s konjskim protistrupom proti modrasovemu strupu [24]. Znaki zastrupitev s strupi navedenih kač so podobni tistim, ki jih povzroči modrasov strup: poškodba tkiva, edem, hemoragija, nekroza tkiva, hipotenzija in motnje v koagulaciji krvi (predvsem slabše strjevanje krvi). Zaščitna učinkovitost paraspecifičnih protistrupov pa v primeru hujših zastrupitev vendarle ni povsem zanesljiva, saj lahko pride do različnih kliničnih zapletov [17]. Razlog tiči v večjih ali manjših razlikah proteinskih sestav strupov. Tako na primer ViperaTAB® (MicroPharm, VB), protistrup, pripravljen na osnovi gadovega strupa, ki se zaradi pomanjkanja specifičnega protistrupa včasih uporablja tudi za zdravljenje zastrupitev z modrasovim strupom, ne nevtralizira toksičnih učinkov vseh komponent slednjega [25]. S poznavanjem podrobne sestave KS lahko izboljšamo protistrupe in prispevamo k bolj učinkovitemu zdravljenju zastrupitev. Bistvene razlike v sestavi strupov modrasa in gada na molekularnem nivoju, ki smo jih razkrili v poglavju 3.1, so dobra osnova za to.

Predmet raziskav, opravljenih v okviru doktorskega dela, sta torej gadov in modrasov strup, njuna sestava in vpliv njunih komponent na srčno-žilni sistem pri človeku.

1.2 Srčno-žilni sistem pri človeku

Srčno-žilni oziroma kardiovaskularni sistem je nujen za oskrbo telesnih organov in tkiv s hranili in kisikom, poleg tega ima pomembno vlogo tudi pri uravnavanju telesne temperature in pri odstranjevanju produktov presnove. Pri človeku srčno-žilni sistem sestavljajo srce in žile, ki jih delimo na arterije, vene in kapilare. Kri, ki se pretaka po žilnem sistemu zaradi delovanja srca, je tekočina, sestavljena iz plazme, v kateri so raztopljeni plazemski proteini, rdečih (eritrocitov) in belih krvnih celic (levkocitov) ter krvnih ploščic (trombocitov).

1.2.1 Srce

Srce je ritmično utripajoči organ, ki poganja kri in hemolimfo po telesu. Ritem za delovanje mu dajejo posebne živčne celice, ki po živčnih snopičih pošiljajo vzburljenje ter tako povzročajo enakomerno krčenje srčne mišice.

Srce ima štiri predelke, dva zgoraj in dva spodaj, in sicer sta zgornja sprejemna, t. i. atrija oziroma preddvora, spodnja pa iztisna, t. i. ventrikla oziroma prekata. Preddvora se odpirata v ventrikla preko atrioventrikularnih zaklopk, ki so prisotne v atrioventrikularnem septumu (pregradi). Desni preddvor in desni prekat skupaj tvorita desno srce, levi preddvor in levi prekat pa levo srce.

Nenormalen srčni ritem imenujemo srčna aritmija – lahko povzroči nenadno smrt ali omotico. Poznamo dva glavna tipa aritmij, in sicer bradikardijo (pod 60 utripov/min) in tahikardijo (nad 100 utripov/min).

S pomočjo elektrokardiograma (EKG) lahko spremljamo potek srčne aktivnosti, kjer je poleg števila utripov na min pomembno tudi, če srce bije enakomerno oziroma ali je moteno prevajanje impulzov v srcu. Osnovni vzorec zapisa enega srčnega impulza je sestavljen iz treh valov: vala P, kompleksa QRS (sestavljeno je iz treh ločenih zobcev, Q, R in S) in vala T.

Električni impulz srčnega cikla se začne v področju desnega preddvora, ki ga imenujemo sinoatrialni vozal. Depolarizacija se nato širi po mišičnih vlaknih preddvorov (P val). Za depolarizacijo je odgovoren vtok Ca^{2+} v celico. Potem sledi kontrakcija preddvorov. Vsak električni impulz iz sinoatrialnega vozla povzroči srčni utrip in določa frekvenco srca. Po končani depolarizaciji preddvorov (traja do 0,10 s), se depolarizacijski val po treh snopih širi proti atrioventrikularnem vozlu, kjer se nekoliko upočasni. Interval P-R predstavlja čas atrioventrikularnega prevoda, ki traja od 0,12 do 0,20 s. Nato depolarizacijski val potuje zelo hitro skozi prevodno tkivo, imenovano Hissov snop, ki se nato deli za vsak prekat (levi in desni krak). Levi krak se dodatno deli na sprednjo in zadnjo vejo. Kraka se razdelita na več manjših vejic (t. i. Purkinijeva vlakna), ki vzdražijo prekata. Na EKG zapisu nastane QRS kompleks. Kompleks QRS odraža hitro depolarizacijo desnega in levega prekata. Prekata imata v primerjavi s preddvoroma več mišičnine, zato ima kompleks QRS običajno znatno višjo amplitudo kot val P. Po depolarizaciji celotnega miokarda se EKG zapis vrne v izolinijo. QRS kompleks ne sme biti širši kot 0,12 s. Na koncu EKG zapisa nastane T val, ki predstavlja vrnitev celičnega membranskega potenciala na začetno vrednost (repolarizacija). Repolarizacija nastane zaradi iztoka K^{+} ven iz celice. Po končani repolarizaciji se ponovno začne depolarizacija in cikel se ponovi [26].

1.2.2 Žile

Krvna žila je cevast organ, po katerem se pretaka kri. Žile so različnih tipov, in sicer: arterije, arteriole, kapilare, venule in vene. Po arterijah (žile odvodnice) teče kri iz srca in v njih je krvni tlak največji. Arteriole imajo manjšo mišično steno od arterij in spreminjajo premer, s tem pa vplivajo na pretok krvi skozi določen predel. Imajo tudi izrazit vpliv na krvni tlak. Arteriole se zaključijo s kapilarami, ki imajo izredno tanko steno in predstavljajo most med arterijami in venami. Kapilarna mreža omogoča izmenjavo hranilnih snovi, plinov, hormonov itd. med krvjo in telesnimi tkivi. Odpadne snovi se preko kapilar iztekajo v venule, te pa nato v vene, ki vodijo nazaj v srce. Vene (žile dovodnice) imajo tanjšo steno od arterij, a praviloma večji premer kot arterije. Kri skozi vene teče počasneje in pod bistveno manjšim tlakom.

Arterije in vene so si po sami anatomske zgradbi zelo podobne. Sestavljene so iz treh plasti: notranje plasti (tunica intima), ki je sestavljena iz endotelijskih celic in notranjega prožnega tkiva; srednje plasti (tunica medija), ki je zgrajena iz mišičnih vlaken in elastičnega tkiva (sestavljeno iz kolagena in elastina); ter zunanje plasti (adventicija), ki jo nadalje razdelimo na zunanje prožno tkivo in plast, sestavljeno iz kolagenskih in prožnih vlaken.

1.2.3 Hemostaza

Hemostaza zajema vse procese, ki ohranjajo kri tekočo in prispevajo k uspešnemu ustavljanju krvavitve ob poškodbi žile ter k regeneraciji le-te. V teh procesih sodelujejo trombociti, komponente žilne stene (endotelijske celice, celice gladkega mišičja in subendotelijske strukture) ter koagulacijski (označeni s črko F in rimsko številko, aktivna oblika pridobi še pripono »a«: FIIa, FVIIa, FVIIIa, FIXa, FXa, FXIa) in fibrinolizni proteini. V normalnih razmerah prevladuje antikoagulacijski mehanizem nad prokoagulacijskimi procesi, saj mora kri v nepoškodovanem ožilju ostati tekoča [27].

Ko pride do poškodbe žile, se proces celjenja rane začne z zoženjem žile, čemur sledi pritrditev trombocitov na poškodovani endotelij. Tako se rana začasno zapre. Hkrati se ob nastanku trombocitnega čepa aktivira tudi koagulacijska kaskada, ki preko niza encimskih procesov privede do nastanka fibrinskega strdka. Koagulacijski faktorji ali faktorji strjevanja krvi so SP, ki se tvorijo v jetrih in so v krvi prisotni kot proencimi. Njihova kaskadna aktivacija privede v končni fazi do aktivacije fibrinogena v netopen fibrin, ki z dodatnim prečnim povezovanjem (omogoča ga FXIIIa) učvrsti trombocitni čep. Ko se žila zaceli, se trombocitno-fibrinski strdek razgradi v procesu fibrinolize [11].

Najtanjše krvne žile, kapilare, so sestavljene le iz ene plasti endotelijskih celic, ki so pritrjene na bazalno membrano, posebno plast ekstracelularnega (zunajceličnega) matriksa. Zunajcelični matriks je sestavljen iz različnih makromolekul, najpogostejše kolagena tipa IV, laminina, nidogena/entaktina in perlekana/heparan sulfat proteoglikanov [28]. Endotelijske celice proizvajajo snovi, ki v odsotnosti poškodbe žile preprečujejo adhezijo in aktivacijo trombocitov ter aktivacijo koagulacijskih proteinov, ob poškodbi pa spojine, ki pripomorejo k aktivaciji tako agregacije trombocitov kot koagulacije [29].

von Willebrandov faktor (vWF) kroži v plazmi in se ob poškodbi žile veže na subendotelijski kolagen. Povečane strižne sile zaradi pretoka krvi in interakcija s kolagenom povzročijo aktivacijo vWF – izpostavijo se vezavna mesta za receptorski kompleks GPIIb–V–IX na površini trombocitov, kamor se potem veže. Tako vWF vzpostavi začetno vezavo trombocitov na mesto poškodbe žile. Vezava trombocitov na mesto poškodbe se nato učvrsti še z neposredno interakcijo teh celic s kolagenom preko receptorjev GPVI in $\alpha_2\beta_1$. Pri tem se trombociti tudi aktivirajo [29]. Aktivirani trombociti spremenijo obliko iz diskoidne v kroglasto s psevdopodiji, s katerimi se pritrdijo na podlago. Iz sekrecijskih zrnč sprostijo molekule, ki aktivirajo sosednje trombocite (na primer tromboksan A₂ (TXA₂) in adenzin difosfat (ADP)), molekule, ki povzročijo konstrikcijo krvne žile (serotonin), molekule, ki sodelujejo pri procesih koagulacije (na primer FV, FXIII, vWF, fibrinogen), ter spojine, ki sodelujejo pri obnavljanju žilne stene. Na plazemski membrani aktiviranih trombocitov se izpostavijo tudi negativno nabiti fosfolipidi, na katere se potem vežejo koagulacijski faktorji in tvorijo komplekse [29]. Na površini trombocitov se ob aktivaciji izrazi receptor – integrin $\alpha_{IIb}\beta_3$ (tudi GPIIb/IIIa), na katerega se vežejo ligandi z vezavnim zaporedjem RGD, kot sta fibrinogen in vWF. Tako se trombociti še dodatno povežejo v čvrst strdek [30].

Nastanek trombocitnega strdka poteka hkrati z aktivacijo koagulacijskih proteinov. V kaskadnem modelu koagulacije krvi se posamezni faktorji aktivirajo v zaporednih reakcijah, ki so razdeljene na dve poti, na intrinzično ali notranjo (sodelujejo: FXIIa, FXIa, FIXa, FVIIIa) ter ekstrinzično ali zunanjo pot (sodelujeta tkivni faktor in FVIIa). Obe poti se združita v t. i. skupno pot (sodelujejo FXa in FVa ter protrombin), ki se zaključi z nastankom fibrinskega strdka [31, 32, 33]. Proces hemostaze je predstavljen na sliki 1.2.

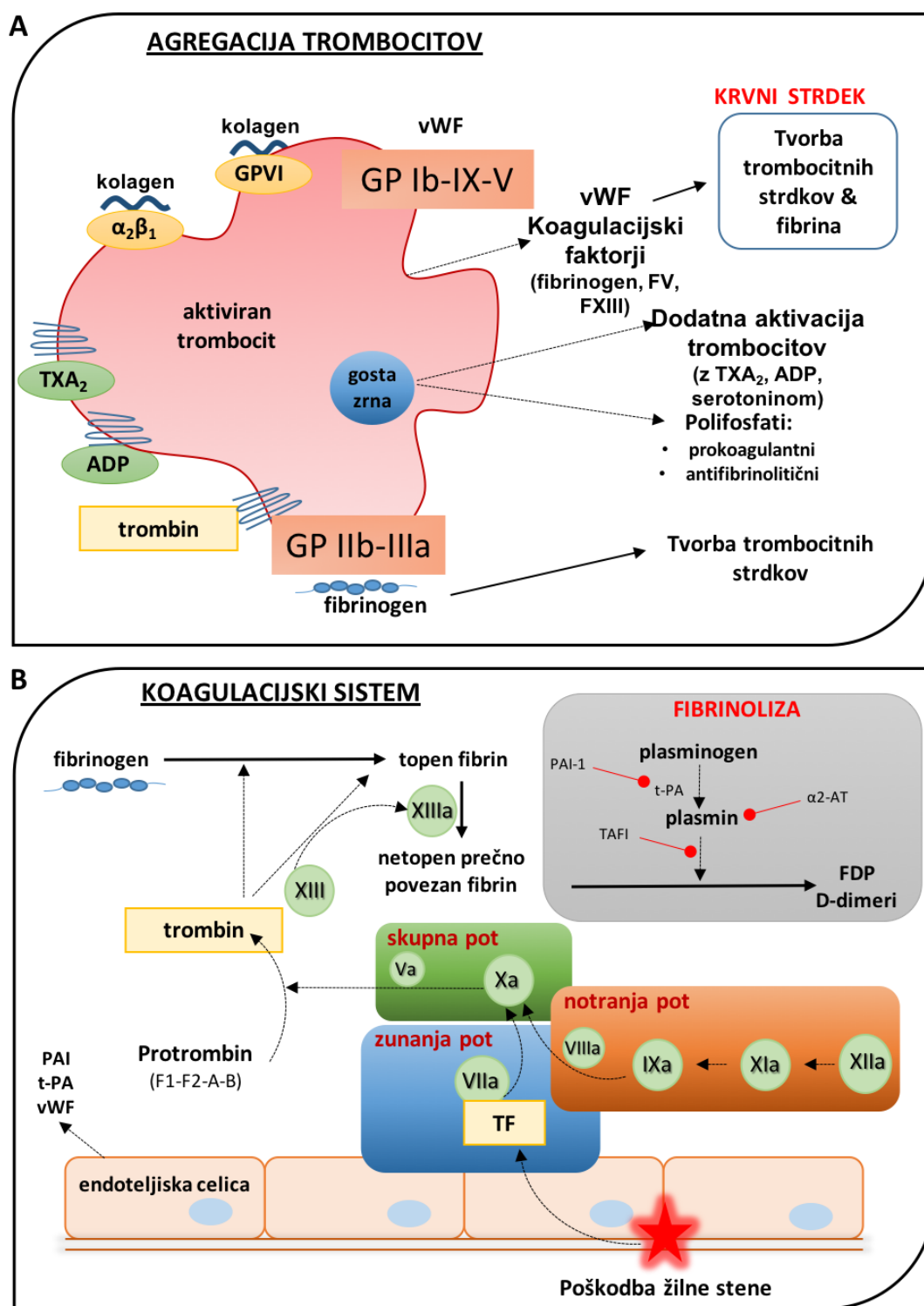
Novejša klinična in eksperimentalna opažanja so pokazala, da kaskadni model ne opiše dogajanj v procesu hemostaze *in vivo* v celoti in da notranja pot nima dejanske fiziološke vloge [34]. Ugotovili so, da je kompleks tkivnega faktorja in FVIIa, ki je prvi korak v zunanji poti kaskadnega modela, glavni sprožitelj hemostaze *in vivo*. Ta opažanja so vključili v novejši opis procesa, ki mu pravimo celični model koagulacije.

Celični model opiše potek koagulacije na sledeči način. Ob poškodbi žilne stene se izpostavi tkivni faktor, ki se izraža na celicah izven ožilja, na primer na fibroblastih. Tkivni faktor je membranski protein in je receptor za plazemsko SP FVII. Ob vezavi na tkivni faktor se FVII najverjetneje aktivira avtokatalitsko. Kompleks FVIIa–tkivni faktor (t. i. ekstrinzični tenazni kompleks) v prisotnosti Ca^{2+} ionov aktivira majhno količino FIX in FX. FV se lahko aktivira s FXa ali z neko nekoagulacijsko proteazo. Na negativno nabitih membranskih fosfolipidih celic, ki nosijo tkivni faktor, se v prisotnosti Ca^{2+} ionov tvori protrombinazni kompleks FXa–FVa, ki katalizira pretvorbo protrombina (FII) v aktivno obliko, α -trombin (FIIa). Majhna količina α -trombina, ki nastane na teh celicah, ima več pomembnih funkcij. Sodeluje pri aktivaciji trombocitov ter na površini trombocitov aktivira FV (sproščajo ga aktivirani trombociti) in FVIII (kroži v plazmi, vezan na vWF) ter FXI. Ob aktivaciji FVIII se FVIIIa in vWF ločita, kar slednjemu omogoči sodelovanje pri adheziji in agregaciji trombocitov [27, 31, 35, 36]. Ko se na mesto poškodbe pritrdi veliko število trombocitov, pride do naslednje, napredujoče faze koagulacije. Koagulacijski faktorji se v prisotnosti Ca^{2+} ionov vežejo na negativno nabite fosfolipide, izpostavljene na površini aktiviranih trombocitov. FIXa se lahko premakne s celic, ki vsebujejo tkivni faktor, na trombocite, kjer tvori kompleks s FVIIIa. FXa pa se ob disociaciji s teh celic hitro inaktivira z zaviralcema TFPI (angl. »tissue factor pathway inhibitor« ali zaviralec poti TF) in antitrombinom. Dodaten FIXa nastane preko aktivacije s FXIa, vezanim na trombocitih. FX, ki je na površini trombocitov, se aktivira s kompleksom FIXa–FVIIIa (t. i. intrinzični tenazni kompleks). Nastali FXa se poveže s FVa, vezanim na trombocite, v protrombinazni kompleks, ki sproži nastanek velikih količin α -trombina. Le-ta potem aktivira fibrinogen, tako da odcepi fibrinopeptida A in B in s tem omogoči polimerizacijo fibrinskih monomerov v fibrinske niti. α -trombin aktivira tudi FXIII, transglutaminazo, ki ustvari kovalentne prečne povezave med fibrinskimi polimeri in utrdi fibrinski strdek [37].

Nastajanje fibrinskega strdka mora biti omejeno, da se ne bi razširilo v okoliške zdrave predele žile. Zato je proces koagulacije reguliran na več načinov [36]:

- s serpini (tj. proteinskimi zaviralci SP), na primer antitrombinom, ki zavira delovanje α -trombina in FXa, mnogo počasneje pa tudi FIXa in FXIa. Inhibitorno aktivnost antitrombina močno ojača vezava na heparin in druge sulfonirane glukozaminoglikane na površini endotelija;
- z aktivacijo proteina C z α -trombinom, ki je vezan na endotelijski receptor trombomodulin. Nastali aktivirani protein C ob pomoči kofaktorja proteina S inaktivira FVa in FVIIIa;
- s TFPI, ki inhibira tako prosti FXa kot FXa, vezan v kompleks tkivni faktor–FVIIa–FXa.

Ko se začne celjenje rane, se fibrinski strdek odstrani v procesu fibrinolize. Glavna endogena fibrinolitična proteaza je plazminogen, ki se v krvi nahaja v neaktivni obliki. Aktivacija plazminogena v aktivni plazmin poteka preko zunanje in notranje poti. Po notranji poti plazminogen aktivirajo urokinaza (angl. »urokinase plasminogen activator«, u-PA) ter faktorji kontaktne aktivacije, FXIIa, visokomolekularni kininogen in kalikrein. Po zunanji poti pa plazminogen aktivira tkivni aktivator plazminogena (angl. »tissue plasminogen activator«, t-PA), ki ga sintetizirajo endotelijske celice. V plazmi je t-PA prisoten v kompleksu z inhibitorjem aktivatorja plazminogena 1 (angl. »plasminogen activator inhibitor-1«, PAI-1). Fibrin močno pospeši katalitsko aktivnost t-PA. Delna razgradnja fibrina s plazminom pospeši vezavo plazminogena na fibrin in tako pospeši fibrinolizo na mestu nastajanja fibrina. Prosti plazmin, ki se sprošča z razgradnjo fibrina, pa hitro zavrejo serpini, α 2-antiplazmin (α 2-AP), PAI-1 in α 2-makroglobulin (α 2-MG) [37, 38].



Slika 1.3: Prikaz sistema hemostaze.
Povzeto po [39].

(A) Primarni odziv na poškodbo krvne žile je agregacija trombocitov, tvorba primarnega trombocitnega strdka na mestu poškodbe žile. Ko se poškoduje endotelij, se izpostavi prokoagulantni subendotelijski matriks, sestavljen iz beljakovin, kot so kolagen, vWF, fibrinogen, laminin in fibronektin. Beljakovine subendotelijskega matriksa se vežejo na glikoproteinske receptorje na površini trombocitov in sprožijo več procesov, (1) adhezijo trombocitov, (2)

aktivacijo trombocitov in (3) tvorbo trombocitnih strdkov. Vezava vWF na trombocitni kompleks GPIb–IX–V ter kolagena na trombocitna receptorja GPVI in integrin $\alpha_2\beta_1$ sproži signal v trombocitu, ki vodi do sproščanja agonistov aktivacije trombocitov, TXA_2 , ADP in serotonina, iz trombocitnih mešičkov. Ti agonisti se skupaj z α -trombinom, produktom koagulacijske kaskade in aktiviranimi trombociti, vežejo na z G-proteini povezane receptorje na trombocitih, kar sproži nadaljnjo aktivacijo trombocitov. Trombocitni mešički vsebujejo tudi polifosfate, ki so tako prokoagulantni kot antifibrinolitični. Aktivirani trombociti sprostijo vWF in koagulacijske faktorje iz celičnih mešičkov, kar vodi v tvorbo fibrina in trombocitnih strdkov. Aktivacija trombocitov izpostavi na celični površini integrin GPIIb/IIIa, ki z vezavo fibrinogena sodeluje pri njihovi agregaciji.

(B) Koagulacijska kaskada – tvorba fibrina. Pri zunanji poti koagulacije poškodba stene žil vodi do izpostavitve tkivnega faktorja na endotelijskih celicah. Tkivni faktor v kompleksu s FVIIa (ekstrinzični tenazni kompleks) aktivira FX v FXa. Zunanjo pot koagulacije zavira TFPI, ki ga proizvajajo endotelijske celice. V intrinzični poti koagulacije (oziroma napredujoči fazi koagulacije) FXIa aktivira FIX v FIXa, ki v kompleksu s FVIIIa (intrinzični tenazni kompleks) aktivira FX v FXa. V skupni poti koagulacije nato nastali FXa tvori protrombinazni kompleks z aktiviranim FVa, ki aktivira protrombin v α -trombin. Protrombin je sestavljen iz treh domen, fragmenta 1, fragmenta 2 in SP (α -trombin). Ob aktivaciji se od protrombina odcepi fragmenta 1 in 2, po cepitvi še ene vezi v predelu SP pa nastane končni produkt α -trombin, ki ga sestavljata disulfidno povezani verigi A in B. α -trombin potem aktivira fibrinogen v fibrin, strukturo pa še dodatno učvrsti FXIII, ki s transglutaminazno aktivnostjo prečno poveže fibrinske niti v fibrinsko mrežo (strdek). V procesu fibrinolize plazmin cepi fibrin v topne razgradne produkte, vključno z D-dimeri. Plazminogen se pretvori v plazmin s pomočjo t-PA, ki ga negativno regulira PAI-1. Aktivnost plazmina zavirata α_2 -AP in s trombinom aktiviran inhibitor fibrinolize.

1.2.4 Srčno-žilne bolezni

Srčno-žilne oziroma kardiovaskularne bolezni prizadenejo srce in/ali krvne žile. Med najpogostejšimi so povišan krvni tlak, koronarna obolenja, srčna kap, bolezen srčnih zaklopk in revmatične bolezni srca. Kardiovaskularne bolezni so glavni vzrok obolenja in prezgodnje smrti ljudi v Evropski uniji in nasploh po vseh delih razvitega sveta. Tovrstne bolezni so tudi vodilni vzrok za umiranje v svetovnem merilu z 31-odstotnim deležem vseh smrtnih primerov [40].

Mnoge izmed kardiovaskularnih bolezni imenujemo tudi bolezni življenjskega sloga, saj se razvijajo skozi čas in so povezane z osebnimi navadami, pomanjkanjem telesne vadbe, nezdravo prehrano, kajenjem in drugimi oblikami načina življenja [41, 42]. Predhodnik večine te bolezni je ateroskleroza, pri kateri se v stenah srednjih in velikih arterij kopičijo majhni ateromatozni plaki. Ateroskleroza je tudi dejavnik tveganja za akutni koronarni sindrom, ki opisuje stanje, za katerega je značilen nenaden primanjkljaj krvi s kisikom v srčnem tkivu. Povezana je tudi s težavami, kot so nastanek anevrizme, tj. izboklin, ki zaradi oslabelosti žilne stene običajno nastanejo na razcepišču možganskih arterij [43].

Razvoj kardiovaskularnih bolezni lahko vključuje tudi nastanek strdka, imenovanega tromb. Ta lahko nastane v venah ali arterijah. Globoka venska tromboza večinoma nastane v venah nog. Ti strdki se lahko odtrgajo z mesta nastanka in potujejo po krvi na drugo mesto v telesu. Tako na primer nastane pljučna embolija, prehodni ishemični napadi ali možganska kap [44]. Tromboze razdelimo glede na žilo, ki jo strdek prizadene, torej na vensko trombozo (na primer globoka venska tromboza, tromboza jetrne vene itd.) in arterijsko trombozo (možganska ter srčna kap). Pri slednji je najpomembnejši razlog za nastanek napredna ateroskleroza, pri kateri se lahko natrga pokrov aterosklerotične obloge in posledično nastane tromb, ki popolnoma zapre že poprej nekoliko zoženo arterijo.

Najpogostejša vzroka smrti kot posledice kardiovaskularni bolezni sta srčna in možganska kap. Na tretjem mestu je venska trombembolija [41, 45]. Ime venska trombembolija združuje pravzaprav dve bolezni: vensko trombozo in pljučno embolijo, do katere lahko pride, ko se tromb odtrga z mesta nastanka v veni (t. i. embolusa), po krvi odplakne v pljuča in tam zamaši pljučno arterijo. V venah je krvni tlak majhen, žilna stena vene je drugačna kot v arterijah, precej tanjša in ni podvržena aterosklerozi. Strdki v venah so največkrat posledica povečane nagnjenosti krvi k strjevanju oziroma upočasnjene krvnega pretoka po veni. Venska tromboza je lahko posledica zgoraj omenjenega življenjskega sloga, lahko pa tudi genetske anomalije, ki se lahko odraža kot a) povišana aktivnost enega ali več faktorjev strjevanja krvi ali inhibitorjev fibrinoliznih proteinov (PAI-1) in/ali b) znižana aktivnost enega ali več antikoagulantnih proteinov (TFPI, antitrombin, aktiviran protein C) in/ali c) prisotnost FV Leiden (gensko spremenjen FV) [46, 47, 48].

Zdravljenje patološkega prekomernega strjevanja krvi poteka z antikoagulantmi, ki neposredno znižujejo aktivnost trombina oziroma inhibirajo njegov nastanek. Tradicionalno zdravljenje poteka z antagonistami vitamina K, med katerimi je najpogosteje uporabljen varfarin [45, 49, 50, 51]. Glavni problemi takega zdravljenja so resni stranski učinki, ki se odražajo v močnih krvavitvah. V izogib težavam, je potreben konstantni nadzor zdravljenja in po potrebi prilagajanje odmerka zdravila. Nekatera od zdravil tudi interagirajo s hrano in drugimi zdravili, kar lahko povzroča še dodatne zaplete. Izboljšave v terapijo venske trombembolije so prinesli novi peroralni antikoagulantni, NOAC (angl. »New Oral AntiCoagulant«), ki selektivno zavirajo delovanje proteinov v koagulacijski kaskadi. Taka zdravila so usmerjena specifično na trombin (dabigatran) ali FXa (apiksaban, edoksaban in rivaroksaban) [52]. Za razliko od tradicionalnih zdravil se učinek NOAC hitro izrazi, njihov farmakološki profil je predvidljiv in ne interagirajo s hrano ali drugimi zdravili. Žal pa trenutno nimajo vsi primerne antidote (le dabigatran ga ima: Praxbind™ tj. idarucizumab) in v primeru, ko pride do predoziranja, lahko pride do nastanka obširne sistemske krvavitve [53, 54, 55].

NOAC si z veliko hitrostjo utrjujejo pot v klinični praksi v Sloveniji, predvsem rivaroksaban in dabigatran [56, 57]. Od leta 2011 sta zdravila odobreni za preprečevanje kapi in sistemskih embolizmov ter za zdravljenje venske trombembolije, od 2013 pa še za preprečevanje aterosklotičnih dogodkov pri odraslih bolnikih z akutnim koronarnim sindromom.

Nasprotje prekomernega strjevanja krvi so hemofilije, kjer se zaradi odsotnosti ali pomanjkanja koagulacijskih faktorjev kri ne strjuje, kot bi se morala. Gre predvsem za genetske anomalije, redkeje pa je težava v tvorbi protiteles proti koagulacijskim faktorjem. Hemofilija A je najpogostejša dedna motnja strjevanja krvi. Pri njej gre za pomanjkanje FVIII. Bolezen se zdravi z intravenoznim dodajanjem FVIII, ki ga pridobijo iz človeške krvne plazme ali s pomočjo proteinskega inženirstva. Pri hemofiliji B gre za pomanjkanje FIX, medtem ko pri hemofiliji C manjka FXI. Von Willebrandova bolezen je druga najpogostejša oblika koagulopatije, pri kateri se tvori premalo vWF, ki sodeluje pri pritrjevanju trombocitov na poškodovano žilno steno. Bolezen se zdravi z dodajanjem humane plazme [58].

Kot že omenjeno, strupi viperid vplivajo na proces strjevanja krvi, povzročajo lokalne poškodbe tkiva, poleg tega pa vplivajo na delovanje srca. Toksini iz teh strupov so tako zanimivi za podrobno karakterizacijo, saj so primerne spojine vodnice za razvoj terapevtskih učinkov za zdravljenje kardiovaskularnih bolezni.

1.3 Komponente kačjih strupov z vplivom na srčno-žilni sistem

Običajno imajo komponente KS, ki so encimsko aktivne, visoko katalitično specifičnost in učinkovitost, poleg tega pa so tako encimsko aktivne kot neaktivne makromolekule tudi toplotno precej stabilne in odporne na razgradnjo s proteinazami [59]. Zato so zelo primerna osnova za razvoj inovativnih farmakoloških učinkovin in/ali diagnostičnih reagentov.

Pot od ustreznega proteina v KS do farmacevtske učinkovine na njegovi osnovi je zelo dolga, obsega pa naslednje pomembne korake: izolacijo ustreznega farmakološko aktivnega proteina iz KS, njegovo fizikalno-kemijsko in biološko karakterizacijo, določitev njegove atomske strukture in določitev molekularnega mehanizma njegovega delovanja [14]. Zbrani podatki se nato uporabijo pri načrtovanju običajno malih kemijskih molekul, ki posnemajo delovanje naravne molekule (mimetik) in so primerni za zdravljenje bolnikov. Poleg terapevtskih učinkovin so proteini iz KS lahko uporabni tudi v diagnostične namene, na primer za klinično testiranje sposobnosti strjevanja krvi [60, 61].

Komponente strupov viperidnih kač, ki so največkrat predmet tovrstnih raziskav, v glavnem sodijo v proteinske družine SP in MP [62]. Primera kačjih toksinov, ki sta našla uspešno uporabo v medicini, sta na primer: SP batroksobin iz strupa navadne suličarke (*Bothrops atrox*), ki se uporablja za defibrinazno terapijo (Defibrase®, Pentapharm, Švica) in za določanje ravni fibrinogena v krvi [60], ter RVV-X – aktivator FX iz strupa Russellovega gada (*Daboia russelii*), ki se uporablja za določevanje lupusnih antikoagulantov v krvi [63].

1.3.1 Komponente kačjih strupov z vplivom na hemostazo in njihova uporaba v kliniki

Na splošno strupi viperid povzročajo motnje strjevanja krvi (hemostaze) kot tudi lokalne poškodbe tkiva (zatekanje, edem, nekrozo tkiva) ter znižanje krvnega tlaka. Komponente teh strupov lahko izzovejo simptome, kot so:

- zmanjšanje sposobnosti strjevanja krvi,
- krvavitve ali hemoragije kot posledica neposredne poškodbe krvnih žil ter
- sekundarne učinke krvavitev (hipovolemični šok in poškodbe notranjih organov) [8].

Vsi omenjeni simptomi izhajajo iz motenj hemostatskega sistema zastrupljenega organizma [9]. V prihodnjih poglavjih bomo opisali nekatere proteine iz družin SP, MP in sPLA₂ iz KS, njihove učinke na hemostazo in izpostavili tiste, ki se klinično uporabljajo. V tabeli 1.1 so predstavljene fiziološke tarče tovrstnih molekul iz modrasovega strupa v srčno-žilnem sistemu in učinek, ki ga kačji proteini povzročijo ob delovanju nanje.

Tabela 1.1: Fiziološke tarče beljakovin, predstavnikov glavnih proteinskih družin v modrasovem strupu, v koagulacijskem sistemu človeka in učinek, ki ga ob tem sprožijo. Povzeto po [11].

Proteinska družina strupne komponente	Tarča v koagulacijskem sistemu človeka	Učinek
SP	trombociti	agregacija
	serpini	inaktivacija
	plazminogen	aktivacija
	fibrinogen	aktivacija
	FII, FV, FVII, FX	aktivacija
	protein C	aktivacija
MP	endotelijske celice	hemoragija
	bazalna membrana	hemoragija
	FX, FII	aktivacija
	trombociti	inhibicija agregacije
	serpini	inaktivacija
	fibrin(ogen)	razgradnja
sPLA ₂	FXa, FIIa, tkivni faktor/FVII	inhibicija
	trombociti	agregacija/ inhibicija agregacije

1.3.1.1 Metaloproteinaze

Zastopanost MP v strupih kač iz družine *Viperidae* je bogata, v nekoliko manjši meri pa so MP prisotne tudi v nekaterih strupih kač iz družin *Elapidae* in *Colubridae* [64]. Skupaj s proteini ADAM (angl. »A Disintegrin And Metalloproteinase«) MP iz KS uvrščamo v poddružino repolizinov (M12B) družine M12 MP, podklan metcinkinov. Vsi metcinkini vsebujejo značilno vezavno mesto za Zn^{2+} , HEXXHXXGXXH, in strukturno ohranjen motiv CIMXP, imenovan Met-obrat. Met v Met-obratu sodeluje pri koordinaciji Zn^{2+} v aktivnem mestu [65].

MP iz KS se sintetizirajo kot večdomenski proencimi, katerih encimsko aktivnost v strupni žlezi zavira t. i. cisteinsko stikalo. Aktivno mesto blokira interakcija med Zn^{2+} in Cys iz ohranjenega zaporedja PKMCGVT v prodomeni. Proteinazno aktivnost MP v strupni žlezi regulirajo tudi citratni ioni, ki kelirajo Zn^{2+} , kisel pH ter kompetitivni tripeptidni inhibitorji (pyroGlu-Asn-Trp, pyroGlu-Gln-Trp ali pyroGlu-Lys-Trp) [66].

Aktivne oblike MP iz KS so glede na organizacijo domen razdeljene v tri razrede, P-I, P-II in P-III [67, 68]. P-I MP (20–30 kDa) vsebujejo le katalitično MP-domeno, P-II MP (30–60 kDa) so sestavljene iz MP- in disintegrinske domene, P-III MP (60–100 kDa) pa iz MP-domene, domene, podobne disintegrinom (D), in domene, bogate s cisteinom (C). Disintegrinska domena v P-II se od domene D v P-III MP razlikuje predvsem v značilnem kratkem vezavnem motivu za integrinske receptorje, ki leži v t. i. disintegrinski zanki. Le-ta v disintegrinski domeni vsebuje zaporedje RGD, v domeni D pa zaporedje XXCD [66]. Nekatalitična domena C vsebuje vezavna mesta za prepoznavo in vezavo različnih substratov, na primer hipervariabilno regijo, tj. predel aminokislinskega zaporedja, v katerem se MP iz KS in ADAM proteini najbolj razlikujejo. Nekatalitični domeni D in C tako z vezavnimi mesti za substrate določata substratno specifičnost posameznega encima. P-II in P-III MP iz KS so glede na posttranslacijske modifikacije, proteolitično procesiranje in oligomerizacijo razdeljene v več podrazredov. P-II MP se največkrat proteolitično procesirajo v MP in disintegrin. Podobno se P-III MP (avto)procesirajo v protein, sestavljen iz D- in C-domene, medtem ko se domena MP razgradi [69]. Strukturno MP iz kačjih strupov v oksidacijskem zunajceličnem okolju stabilizirajo številne disulfidne vezi. Razporeditev Cys v aminokislinskih zaporedjih MP iz KS je ohranjena znotraj posameznih razredov teh proteinov [69].

1.3.1.1.1 Patofiziološka aktivnost metaloproteinaz

MP iz KS imajo širok nabor patofizioloških aktivnosti. Povzročijo lahko hemoragijo, edem, lokalno mionekrozo, trombogenezno, znižujejo krvni tlak, odgovorne pa so lahko tudi za povzročanje vnetnih procesov [70].

MP iz KS povzročajo krvavitve, tako da cepijo nekatere gradnike bazalne membrane, na primer laminin, kolagen tipa IV, nidogen, fibronektin in perlekan [70]. Med hemoragično aktivnimi MP iz KS imajo P-III MP najizrazitejši hemoragični učinek, hkrati pa poleg lokalne povzročajo tudi sistemsko krvavitev. Vzroki za to so: i) domeni D in C, ki usmerjata in omogočata vezavo P-III MP na proteine bazalne membrane ter integrine na endotelijskih celicah; ii) ti dve domeni omogočata tudi interakcijo MP s komponentami hemostatskega sistema, kot so trombociti, vWF in fibrinogen, in na tak način preprečujeta strjevanje krvi, kar povečuje hemoragični učinek MP; iii) $\alpha 2$ -MG učinkovito inhibira P-I MP, medtem ko P-III MP ne [71].

Poškodbe endotelija, ki jih ob zastrupitvi povzročajo strupne MP, imajo lahko veliko širše posledice kot le povzročitev lokalne krvavitve. Ker na mestu ugriza pride do poškodb kapilar in krvavitve, to posledično vodi v pomanjkanje dotoka krvi, s tem pa kisika in hranil v okoliško tkivo (ishemija) in celično smrt (nekrozo). Če je pretok krvi prekinjen dalj časa, je motena tudi regeneracija skeletnih mišic, kar povzroči fibrozo in trajno izgubo tkiva [72].

Prvi hemostatski učinek MP iz KS, ki so ga raziskovalci opazili in opisali, je bila cepitev fibrin(ogen)a. Fibrin(ogen)olitične MP so izolirali iz strupov elapid in viperid [5]. Fibrinogen je topna molekula, ki se po cepitvi s trombinom pretvori v netopen fibrinski polimer, kar je glavna sestavina krvnih strdkov. Fibrinogen je dimeren protein, ki se sintetizira v jetrih. Sestavljen je iz treh parov polipeptidnih verig (verige $\alpha\alpha$, $\beta\beta$ in γ), ki so medsebojno povezane z disulfidnimi vezmi [37]. MP iz KS na fibrin(ogen) delujejo neposredno in za svojo aktivnost ne potrebujejo nobenih kofaktorjev ali encimov iz krvi. Običajno cepijo $\alpha\alpha$ - in/ali $\beta\beta$ -verigo fibrinogena na C-koncu molekule [73]. Večina nehemoragičnih fibrinogenolitičnih MP iz KS spada v P-I razred in prednostno cepi $\alpha\alpha$ -verigo, t. i. α -fibrinogenaze. Razgradni produkti fibrinogena se sicer lahko aktivirajo s trombinom, ne morejo pa tvoriti visoko polimeriziranih stabilnih agregatov, zato je učinek fibrinogenolitičnih MP antikoagulanten. Nekatere MP iz KS imajo poleg fibrinogenolizne tudi fibrinolizno aktivnost [61].

Strupi viperid vsebujejo tudi prokoagulantne MP. Te molekule aktivirajo FX ali protrombin in tako skrajšajo čas koagulacije. MP iz KS, aktivatorji FX za svojo aktivnost potrebujejo Ca^{2+} ione tako kot naravni aktivatorji in tudi cepitveno mesto v FX je enako kot pri slednjih [74]. Aktivatorji protrombina iz KS so na osnovi strukturnih lastnosti in potrebe po kofaktorjih razdeljeni v štiri skupine (A – D). Aktivatorji iz skupin A in B so MP iz KS, medtem ko sta C in D SP iz KS. Prvi ne potrebujejo nobenih kofaktorjev za svoje delovanje, medtem ko drugi potrebujejo Ca^{2+} ione. MP iz KS najprej cepijo vez $\text{Arg}^{320}\text{-Ile}^{321}$ v protrombinu in ga spremenijo v meizotrombin, ki se nato avtokatalitsko spremeni v trombin [74].

Znane so tudi plazminogenolizne MP iz KS, pri čemer se po cepitvi plazminogena sproščajo angiostatinom podobne molekule, ki zavirajo proliferacijo žilnih endotelijskih celic in s tem celjenje poškodovanega tkiva [12].

MP iz KS vplivajo tudi na agregacijo trombocitov, tako da zavirajo njihovo interakcijo s kolagenom in/ali vWF ter fibrinogenom. Pri tem so tarča sami ligandi in/ali njihovi receptorji na membrani trombocitov, integrini $\alpha_2\beta_1$, GPVI, $\alpha_{IIb}\beta_3$ in GPIIb. Nekatere MP iz KS cepijo tudi vWF in s tem zmanjšujejo njegovo sposobnost, da interagira s trombociti ali veže kolagen [74].

V modrasovem strupu je bilo do sedaj opisanih več MP, vse sodijo v razred P-III: nehemoragična amoditaza, fibrolaza, ki cepi tudi $\alpha\alpha$ -verigo fibrinogena [75]; hemoragini VaH1, VaH2, VaH3 [76], VaH4 [77] in amoditagin; dva aktivatorja FX, VaFXA-I in VaFXA-II-III [78]. Strup gada prav tako vsebuje aktivatorje FX, in sicer MP z molekulsko maso 38 kDa in homodimerno MP s podenotama velikosti 59 kDa [79], vendar njuni aminokislinski zaporedji nista znani.

V sekciji 3.4 je podrobno opisana še ena P-III MP iz modrasovega strupa, ki smo jo analizirali v okviru tega doktorskega dela. Je nehemoragična in fibrinogenolitična.

1.3.1.1.2 Uporaba metaloproteinaz v kliniki

MP iz KS imajo širok spekter bioloških aktivnosti, zato se jih pogosto uporablja tudi v diagnostične namene.

Ekarin, P-III MP iz strupa puščavske puce (*Echis carinatus*), je aktivator protrombina iz skupine A, ki nima vpliva na ostale koagulacijske faktorje. Določanje ekarinskega časa je laboratorijski test (Pentapharm, Švica), ki se uporablja za spremljanje koncentracije antikoagulantov hirudina in nekaterih NOAC-ov [80]. Ekarin se v kombinaciji s tekstarinom, aktivatorjem protrombina iz skupine D iz navadne rjave kače (*Pseudonaja textilis*), uporablja za določitev lupusnih antikoagulantov [81].

Strup Russellovega gada vsebuje že omenjeno MP, t. i. RVV-X, P-III MP, ki aktivira FX [82]. Uporablja se v mnogih metodah določanja plazemske koncentracije FX za razlikovanje med pomanjkljivostmi FVII in FX in pri določevanju lupusnih antikoagulantov, kjer se namesto izoliranega RVV-X uporablja razredčen strup Russellovega gada (dRVVT = 'Diluted Russell's viper venom time') [83, 84].

Tudi fibrinolitične MP iz KS brez hemoragične aktivnosti so pritegnile pozornost raziskovalcev zaradi možne klinične uporabe pri raztapljanju trombov, ker jih ne inhibirajo serpi v krvi in delujejo direktno, tj. ne aktivirajo plazmina oziroma endogenega fibrinoliznega sistema in zato je manj zapletov, kot so krvavitev, reokluzija žil in trajna poškodba tkiva [73]. Na osnovi fibrinolitične MP fibrolaze iz strupa bronastega glaveža (*Agkistrodon contortrix contortrix*) so razvili rekombinantni encim alfineprazo, ki je prišla v III. fazo kliničnih testiranj kot trombolitična učinkovina [85, 86].

1.3.1.2 Fosfolipaze A₂

PLA₂ specifično katalizirajo hidrolizo estrske vezi na mestu *sn*-2 v glicerofosfolipidih in tako se tvorijo lizofosfolipidi in proste maščobne kisline. V družini PLA₂ so encimi glede na svoje strukturne lastnosti razvrščeni v 16 skupin (GI do GXVI) in v številne podskupine [87]. V KS so našli PLA₂ iz skupin GI in GII, ki sodijo med sekretorne PLA₂ (sPLA₂) [88]. GI sPLA₂ najdemo v strupih elapidnih kač, predstavnice skupine GII pa v strupih viperidnih kač [89]. sPLA₂ so proteini velikosti 14–18 kDa s petimi do osmimi disulfidnimi vezmi. V aktivnem mestu imajo katalitično diado His/Asp in za katalizo potrebujejo Ca²⁺ ione [88, 90]. Primarna struktura vseh sPLA₂ je 40–99 % identična, kar pomeni, da je tudi njihova tridimenzionalna struktura zelo podobna. Kljub temu pa te molekule izražajo zelo različne farmakološke aktivnosti.

1.3.1.2.1 Patofiziološka aktivnost fosfolipaz A₂

sPLA₂ lahko na organizem učinkujejo nevrotoksično, kardiotoksično, miotoksično, hemolitično, hipotenzivno ali antikoagulantno. Poleg tega lahko vplivajo tudi na agregacijo trombocitov. Velika raznolikost patofizioloških aktivnosti je posledica tako razlik v encimski aktivnosti kot tudi sposobnosti vezave na specifične (gliko)proteinske ali (gliko)lipidne receptorje v različnih tkivih in organih. V primerih, ko se sPLA₂ veže na tarčni receptor, lahko mehanizem delovanja vključuje fosfolipazno aktivnost ali pa je od le-te neodvisen. V primeru encimsko odvisnega delovanja lahko farmakološki učinek povzroči sama hidroliza fosfolipidov ali pa nastali produkti hidrolize [13, 88, 91].

Nekatere sPLA₂ iz KS se lahko z visoko afiniteto vežejo na FXa in s tem preprečijo nastanek protrombinaznega kompleksa [92, 93]. Kot indirektni inhibitorji FXa tako predstavljajo obetavno smer razvoja antikoagulantnih spojin. Antikoagulantno delovanje sPLA₂ je tako večinoma posledica vezave na proteinske receptorje in ne na fosfolipide [92, 94].

sPLA₂ z vplivom na agregacijo trombocitov razdelimo na tri skupine. V prvi skupini so tisti encimi, ki inducirajo agregacijo trombocitov, v drugi pa tisti, ki ta proces inhibirajo. sPLA₂ iz tretje

skupine imajo dvofazni učinek, pri nizkih koncentracijah ali pri kratkih časih inkubacije povzročijo agregacijo trombocitov, pri daljšem času inkubacije ali pri visokih koncentracijah pa agregacijo zavirajo. Mehanizmi, kako sPLA₂ vplivajo na agregacijo, niso razjasnjeni, vendar študije kažejo, da vključujejo encimsko aktivnost [12, 95].

V modrasovem strupu so bile do sedaj opisane sledeče sPLA₂: amoditoksini (Atx) A, B in C; amoditina (Atn) I₁ in I₂ – nevtralna encima brez toksične aktivnosti [96] in AtnL. Atx so presinaptično nevrotoksične bazične sPLA₂, ki inhibirajo sproščanje acetilholina iz perifernih nevronov v živčno-mišični stik ali sinapso, do česar pride v treh fazah. Najprej prehodno inhibirajo sproščanje nevrotoksinov acetilholina iz živčnega končiča motoričnega nevrona, nato v drugi fazi, prav tako prehodno, povečajo njegovo sproščanje v sinaptično režo. V tretji fazi sledi nepovratna blokada sproščanja acetilholina, posledica pa je mišična paraliza. Nevrotoksičnost Atx je odvisna od vezave na različne proteine, ki so prisotni na plazemski membrani živčnega končiča, v endoplazemskem retikulumu, v citosolu oziroma v mitohondrijih živčne celice [91]. AtxA ima poleg nevrotoksičnega tudi antikoagulantni učinek, do katerega pride po njegovi vezavi na FXa, kar povzroči inhibicijo delovanja protrombinaznega kompleksa [93].

AtnL je encimsko neaktiven homolog AtxA, ki deluje na organizem miotoksično [97]. Miotoksičnost pri sPLA₂ je pogojena z vezavo na lipide in verjetno tudi proteinske komponente v plazemski membrani mišične celice. Po vezavi miotoksične sPLA₂ poškodujejo plazemske membrane mišičnih celic, lahko s svojo encimsko aktivnostjo, lahko pa tudi neodvisno od nje. V obeh primerih pride do obsežnega vdora Ca²⁺ v mišično celico, kar sproži niz s Ca²⁺-pogojenih degenerativnih dogodkov, na primer aktivacijo citosolnih proteaz, kalpainov, in od Ca²⁺ odvisnih citosolnih PLA₂. Prav tako je dvig Ca²⁺ usoden tudi za nemoteno delovanje mitohondrijev. Nekatere miotoksične sPLA₂ vplivajo na diferencirana mišična vlakna na selektiven način, medtem ko druge kažejo širši citolitični učinek [98].

1.3.1.3 Serinske proteaze

SP so sestavni del strupov kač iz družin *Viperidae*, *Colubridae* in *Elapidae* [99, 100]. SP iz KS spadajo v klan PA, poddružino S1A tripsinu-podobnih SP [101, 102]. V aktivnem mestu imajo značilno katalitično triado His-Asp-Ser. Peptidne vezi cepijo tako z isto specifičnostjo kot tripsin, na C-koncu pozitivno nabitih aminokislin. Vsebujejo 12 Cys, od katerih jih 10 tvori disulfidne vezi na enak način, kot se te tvorijo pri tripsinu. Preostala dva Cys tvorita disulfidni mostiček, ki je značilen za SP iz KS. SP iz KS se sintetizirajo kot proencimi, dolgi približno 260 aminokislin, ki imajo na N-koncu 18 aminokislin dolg signalni peptid in 6 aminokislin dolg propeptid. Zrele oblike so praviloma enoverižni glikoproteini, katerih molekulska masa se giblje med 26 in 67 kDa in je odvisna od količine N- ali O-vezanih sladkorjev [103]. Kljub 50–80 % identičnosti v aminokislinskem zaporedju so SP iz KS encimi z zelo raznolikim patofiziološkim delovanjem, saj lahko vplivajo na agregacijo trombocitov, koagulacijo krvi, fibrinolizo in krvni tlak [104, 105].

1.3.1.3.1 Patofiziološka aktivnost serinskih proteaz

V KS je bilo opisanih že vrsto SP z izrazitim vplivom na proces strjevanja krvi. SP iz KS, imenovane trombinu podobni encimi (angl. »thrombin-like enzymes«, TLE), sprožijo polimerizacijo fibrinogena podobno kot α -trombin, čeprav imajo z α -trombinom le ~33 % identično primarno strukturo [106]. Inhibitorji α -trombina, kot so antitrombin, heparin in hirudin, ponavadi ne zavirajo delovanja TLE. TLE so razdeljeni na tri skupine glede na to, ali odcepijo fibrinopeptid A iz A α -verige, fibrinopeptid B iz B β -verige fibrinogena ali oba tako kot α -trombin [107].

Fibrino(geno)litične SP iz KS neposredno razgrajujejo fibrinske strdke in/ali preprečujejo nastajanje strdka z razgradnjo fibrinogena. V večini prednostno cepijo B β -verigo fibrinogena in imajo manjši učinek na A α -verigo [73].

SP iz KS, ki aktivirajo protrombin, so strukturni in funkcionalni homologi koagulacijskega faktorja FXa oziroma protrombinaznega kompleksa FXa–FVa [104]. Najdemo jih le v avstralskih elapidah (strupenih gožih) in sodijo v skupini C in D protrombinskih aktivatorjev iz KS (skupni A in B sta opisani v poglavju 1.3.1.1.1). Aktivatorji skupine C za svoje delovanje potrebujejo Ca^{2+} ione in negativno nabite fosfolipide, pripadniki skupine D pa še FVa. Obe skupini cepita protrombin na enakih mestih kot FXa.

Do zdaj sta med SP iz KS znana le dva aktivatorja FX [74]. Izolirana sta bila iz strupa kraljeve kobre (*Ophiophagus hannah*) [108] in progastega krajta (*Bungarus fasciatus*) [109]. Sta enoverižna glikoproteina z molekulsko maso 62 oziroma 70 kDa, njuno aminokislinsko zaporedje ni znano, aktivnost pa je odvisna od Ca^{2+} ionov.

V KS so bile opisane tudi SP, ki aktivirajo FV. To sta enoverižni 28,4 kDa VLFVA iz levantinskega gada (*M. lebetina*) [110] in 26 kDa RVV-V iz strupa Russellovega gada [111], ki aktivirata FV s cepitvijo ene same peptidne vezi (Arg^{1545}).

Številne SP iz KS aktivirajo protein C na enak način kot α -trombin, a pri aktivaciji ne potrebujejo trombomodulina. Druge lahko aktivirajo plazminogen, inaktivirajo endogene zaviralce SP ali sprožijo agregacijo trombocitov [99].

SP iz KS, ki vplivajo na krvni tlak, so kalikreinu podobni encimi, ki sproščajo hipotenzivna peptida bradikinin in Lys-bradikinin iz kininogenov v plazmi sesalcev. Nekatere izmed teh SP razgrajujejo tudi angiotenzin, polipeptidni hormon, vključen v renin-angiotenzinski sistem, ki uravnava prostornino zunajcelične tekočine in krvni tlak [112].

Do sedaj so bile opisane le tri SP iz modrasovega strupa, in sicer dva kalikreinu podobna encima ter fibrinogenaza z neobičajno katalitično diado, imenovana VaSP1, ki lahko cepi tudi protrombin, FX in plazminogen [113, 114].

S doktorskim delom smo dopolnili opis SP iz modrasovega strupa s še dvema molekulama, encimsko neaktivnim homologom SP močno antikoagulantnega delovanja (sekcija 3.5) in SP, ki aktivira tako FV kot FX (sekcija 3.6).

1.3.1.3.2 Uporaba serinskih proteaz v kliniki

SP iz KS so že vrsto let uporabno diagnostično orodje za določanje koagulacijskih časov krvi in nepravilnosti v sestavi krvi. V nadaljevanju so naštetje le najbolj pomembne.

ACC-C je SP iz strupa mokasinke (*Agkistrodon contortix*), ki z visoko učinkovitostjo aktivira protein C. Za razliko od α -trombina njeno delovanje ni odvisno od trombomodulina. Produkt na osnovi te SP, Protac® (Pentapharm, Švica), se uporablja pri diagnosticiranju pomanjkljivosti proteinov C in S ter napak v antikoagulantni negativni povratni zanki proteina C [115, 116].

RVV-V je SP iz strupa Russellovega gada, ki aktivira FV s cepitvijo za Arg^{154} in je odporna na inhibicijo z antitrombinom. Uporablja se pri rutinskem določanju plazemske koncentracije FV in pri ugotavljanju mutacije FV, imenovane mutacija Leiden, kjer je Arg^{506} zamenjan z Glu. Aktiviran protein C ne more inaktivirati FVa Leiden, kar posledično vodi v prekomerno strjevanje krvi. FV Leiden na primer odkrijejo kar pri petini bolnikov z vensko trombozo. Eden od testov za določanje te mutacije (Pefakit®, Pentapharm, Švica) vključuje tudi noskarin, aktivator protrombina iz strupa tigrovke (*Notechis scutatus*) [117].

Haempatch™ (QRxPharma, Avstralija) je rekombinantna oblika SP podobne FXa na osnovi SP podenote aktivatorja protrombina iz skupine C iz strupa navadne rjave kače (*P. textilis*). Tako *in vitro* kot *in vivo* v živalskih modelih ta produkt povzroči koagulacijo krvi neodvisno od Ca^{2+} ionov, celo učinkoviteje od α -trombina. Haempatch™ je trenutno v predkliničnih študijah kot prokoagulantna učinkovina za preprečevanje krvavitev pri kirurških posegih.

Nekatere SP iz KS se tudi že uporabljajo v terapevtske namene, na primer Viprinex® in Defibrase®. Viprinex® (Ancrod, Nemčija) je fibrinogenaza iz strupa malajske jamičarke (*Calloselasma rhodostoma*), ki se uporablja za defibrinogenizacijo krvi [118]. Podobno delovanje ima tudi produkt Defibrase® (Pentapharm, Švica), ki je zdravilo, narejeno na osnovi SP

batroksobin iz strupa navadne suličarke (*B. atrox*) [119].

1.3.2 Komponente z vplivom na srce

Kardiotoksične komponente so predvsem prisotne v strupih kač iz družine *Elapidae* in *Atractaspidae*. Pri delovanju kardiotoksinov iz KS so bila zabeležena različna odstopanja od normalne EKG slike, ki je predstavljena v poglavju 1.2.1. V strupu kače mrtvomorskega zemeljskega gada (*Atractaspis engaddensis*) so identificirali kardiotoksične peptide dolžine 21 AK, sarafotoksine [120]. Sarafotoksini so v miškah izzvali spremembe v EKG, na primer: povečanje amplitude vala R, prehodno zvišanje segmenta ST ter podaljšanje intervala PR, ki se je razvilo v atrioventrikularni blok, prekinitev živčne komunikacije med atrijema in srčnimi prekat. V strupu kobre (*Ophiophagus hannah*) so identificirali sPLA₂, ki je povzročila spremembe v EKG sliki in tahikardijo [121].

V KS so odkrili že vrsto molekul z različnim delovanjem na srčno mišico [122]. Kardiotoksične komponente v strupih kober povzročajo depolarizacijo in nekrozo srčne mišice. Gre za triprstne toksine po strukturi, ki povzročijo depolarizacijo celične membrane z interakcijo z membranskimi encimi (protein kinazo C, Na⁺/K⁺ ATPazo) ali z vezavo na integrine oziroma proteine, ki se vežejo na K⁺ kanalčke [122]. Kardiotoksin taicatoksin, izoliran iz strupa avstralskega taipana (*Oxyuranus scutellatus*), je specifičen zaviralec delovanja Ca²⁺ kanalov v srčni mišici [123].

Kot že omenjeno v poglavju 1.3, so pri zastrupitvah z gadovim strupom zasledili tudi toksične učinke na srce [21]. Opazili so na primer atrioventrikularni blok druge stopnje, bradi/tahiaritmije, atrijsko fibrilacijo in celo miokardni infarkt (srčno kap).

Redkeje je bila kardiotoksičnost opisana pri pacientih po zastrupitvi z modrasovim strupom [22, 23]. V raziskavi Lukšiča in sod. (2006) so karakterizirali vpliv modrasovega strupa na izolirano podganje srce s pomočjo t. i. Langendorffovega modela. Langendorffov model izoliranega in perfuziranega podganjega srca omogoča širok spekter meritev srčnih funkcij. Poskusi so ponovljivi in razmeroma poceni ter kljub nekaj pomanjkljivostim predstavljajo zelo koristno orodje v sodobnih kardiovaskularnih in farmakoloških raziskavah [124]. Pri visoki dozi strupa je prišlo v nekaj min do ireverzibilnega asistoličnega srčnega zastoja v hiperkontraktilnem stanju. S tem so potrdili prisotnost kardiotoksičnih molekul, tudi v strupu modrasa. Podobne učinke na izoliranem podganjem srcu so opazili tudi pri strupih levantinskega gada (*M. lebetina*) in nosorogega gada (*Bitis nasicornis*) [125, 126].

Poznavanje in razumevanje mehanizma delovanja kardiotoksičnih komponent KS je pomembno za pravilno zdravljenje pacientov po zastrupitvi.

Poglavje 2

Namen doktorskega dela in hipoteze

Glavni namen moje doktorske disertacije je bilo iskanje in karakterizacija molekul iz strupov modrasa in gada, ki bi bile primerne za razvoj novih terapevtskih učinkovin za zdravljenje motenj hemostaze in funkcij srčno-žilnega sistema ali pa bi bile uporabne v diagnostične namene v hematologiji.

Ob zastrupitvi z modrasovim strupom so pri človeku izraženi predvsem hemoragija, edem in nekroza tkiva. Zastrupitev človeka z gadovim strupom pa povzroči zatekanje (edem) v okolici ugriza, lahko tudi širše, in anemijo, povezano s krvavitvijo in hemolizo. Omenjeni simptomi so bolj izraziti pri zastrupitvi z modrasovim strupom kot z gadovim, kar kaže tako na razliko v njuni sestavi kot tudi v vsebnosti posameznih vrst toksinov. Z namenom razkrivanja teh razlik smo se odločili s pomočjo proteomike primerjati proteinski sestavi obeh strupov med seboj ter z uporabo različnih kliničnih testov strjevanja krvi primerjati prokoagulantno in antikoagulantno aktivnost modrasovega in gadovega strupa.

Posebno pozornost smo se namenili usmeriti na tiste komponente modrasovega strupa, ki bi izražale zelo močno pro- ali antikoagulacijsko aktivnost. Razviti smo želeli postopke za izolacijo hemostatsko aktivnih molekul iz strupa v čisti in aktivni obliki. Molekulam, ki bi bile visoko tarčno specifične, smo se namenili določiti tudi molekularni mehanizem delovanja ter identificirati dele molekularne strukture, ki so odgovorni za farmakološko aktivnost. Prav ti deli strukture so namreč najbolj pomembni kot platforma za oblikovanje novih zdravilnih učinkovin.

Komponente viperidnih strupov s kardiotoksičnim delovanjem doslej še niso bile opisane na molekularnem nivoju, zato je bil naš namen, da jih v strupu modrasa ali gada identificiramo, s tem pa prispevamo k izboljšanju terapije zastrupitev s strupi viperidnih kač.

2.1 Hipoteze

1. Komponente modrasovega in gadovega strupa vplivajo na koagulacijo krvi kot tudi na srčno-žilni sistem pri človeku. Strupa obeh lokalnih viperid predstavljata ustrezen vir za izolacijo molekul z omenjenimi aktivnostmi. Proteomska primerjava strupov in njihovih učinkov na hemostazo lahko poleg identifikacije najbolj aktivnih komponent na srčno-žilni sistem pripomore tudi k izboljšanju seroterapije zastrupitve po modrasovem oziroma gadovem ugrizu.
2. Modrasov strup vsebuje α -fibrinogenazo, ki ima terapevtski potencial kot učinkovina za defibrinogenacijo.
3. Modrasov strup vsebuje proteine, ki zavirajo notranjo pot koagulacije krvi. Ti proteini so primerni kot spojine vodnice za razvoj bolj varnih antikoagulantov za zdravljenje venske tromboembolije, ki je glavni vzrok za nastanek dveh resnih srčno-žilnih bolezni, tromboze globokih ven in pljučne embolije.

Poglavje 3

Znanstvene publikacije

Doktorsko delo je sestavljeno iz šestih znanstvenih publikacij, od katerih je vseh šest publikacij že objavljenih. Sledijo si glede na zaporedje hipotez v poglavju 2. Prva hipoteza se nanaša na primerjavo sestave in hemostatske aktivnosti modrasovega in gadovega strupa, kar obravnava naša prva znanstvena publikacija (sekcija 3.1). Glavne razlike med omenjenima strupoma so v prisotnosti oziroma odsotnosti nevrotoksičnih komponent. Te smo našli le v modrasovem strupu. Prav tako je v modrasovem strupu prisotnih več komponent, ki povzročajo trombocitopenijo (zmanjšano število trombocitov) in vplivajo na strjevanje krvi kot v gadovem strupu. Iz tega razloga smo modrasov strup izbrali kot vir za iskanje novih hemostatsko aktivnih molekul, ki jih opisujemo v nadaljevanju. Znanstvena publikacija, predstavljena v sekciji 3.1, podrobneje predstavi tudi razloge, zakaj protistrup, pripravljen s pomočjo gadovega strupa, ni dovolj učinkovit za zdravljenje resnejših zastrupitev z modrasovim strupom. Obema strupoma je skupno kardiotoksično delovanje, vendar je bilo to večkrat klinično opisano v primeru zastrupitve z gadovim strupom. Kaj daje strupoma obeh viperid tudi kardiotoksični značaj, smo opisali v publikacijah, predstavljenih v sekcijah 3.2 in 3.3.

Modrasov strup vsebuje proteine, ki zavirajo koagulacijo krvi. V doktoratu smo opisali dva nova antikoagulantna proteina, VaF1 in *VaaSPH-1*. Na našo hipotezo s tega področja odgovarja znanstvena publikacija, ki jo predstavljam v sekciji 3.4. Ta opisuje izolacijo in biokemijsko karakterizacijo VaF1, ki sodi med MP razreda P-III. VaF1 je nehemoragična molekula, izraža pa fibrinogenolizno aktivnost kot tudi sposobnost cepitve nekaterih drugih proteinov v koagulacijski kaskadi. S tem ko jih le cepi in pri tem ne aktivira, deluje antikoagulantno.

VaaSPH-1 je za razliko od VaF1 encimsko neaktivna molekula. Kljub temu izrazito zavira koagulacijo krvi po notranji poti. Strukturno je sorodna med SP iz KS, ki je najbolj zastopana, a le slabo raziskana družina proteinov v strupu modrasa. Na tretjepostavljeno hipotezo odgovarjamo v znanstveni publikaciji, predstavljeni v sekciji 3.5. V tem delu je predstavljena izolacija in biokemijska karakterizacija ter možna uporaba *VaaSPH-1* kot spojine vodnice v pripravi inovativnega in varnega antikoagulacijskega zdravila.

V postopku izolacije *VaaSPH-1* smo izolirali tudi SP, ki ima za razliko od *VaaSPH-1* prokoagulanten učinek na kri. Podrobno smo to molekulo opisali v članku, ki je predstavljen v sekciji 3.6. Molekulo smo poimenovali *VaaSP-VX* po tem, ker je verjetno sposobna aktivirati tako koagulacijski FV kot tudi FX. Z močnim in specifičnim vplivom na potek strjevanja krvi je ta molekula zanimiva tako za diagnostično kot tudi za terapevtsko uporabo v primeru hemofilije. Tudi rezultati tega dela potrjujejo našo prvo hipotezo, da je modrasov strup primeren vir proteinov z izrazitim vplivom na strjevanje krvi in zato primeren vir proteinov za uporabo ali razvoj substanc za diagnostiko in terapijo bolezni krvi.

3.1 Znanstvena publikacija 1: Proteomika gadovega strupa za pojasnitev razlik v patologiji, ki jo povzroči zastrupitev z modrasovim strupom: terapevtske posledice

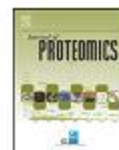
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Zaradi različnih kliničnih manifestacij pri zastrupitvah z modrasovim ali gadovim strupom je bilo treba sistematično raziskati, v čem se razlikujeta omenjena strupa na molekulskem nivoju. Namreč ugriz modrasa lahko povzroči sistemsko hemoragijo in velik učinek na hemostazo, kar je v primeru *Vbb*, tj. gada, manj izrazito. Prav tako je nevrotoksičen učinek na človeka izrazit po zastrupitvi z modrasovim strupom, po zastrupitvi z gadovim pa zelo redek. Tudi naši *ex vivo* poskusi so potrdili klinična opažanja. Gadov strup je za razliko od modrasovega deloval predvsem miotoksično na izolirani mišji živčno-mišični preparat. Poleg tega je bil manj učinkovit od modrasovega pri zaviranju koagulacije ter agregacije in aglutinacije trombocitov, sprožene z ADP in kolagenom oziroma ristocetinom.

V proteomski študiji, ki je vključevala ločevanje kompleksnega KS na številne frakcije in podfrakcije, izolacijo posameznih komponent ter pridobivanje delov sekvenc in njihovo primerjavo z različnimi bazami aminokislinskih zaporedij proteinov, smo ugotovili glavne razlike ter podobnosti v sestavi obeh strupov. Proteomska analiza je pokazala, da vsebuje gadov strup manjše število komponent od modrasovega oziroma da je veliko manj kompleksen. V modrasovem strupu je predvsem več nizkomolekulskih proteinov z navidezno molekulsko maso med 10 in 20 kDa, kamor sodijo disintegrini in sPLA₂. Poleg tega je le-ta bogatejši s komponentami z navidezno molekulsko maso nad 50 kDa, kamor sodijo hemoragične MP. V obeh strupih pa so prisotni predstavniki proteinskih družin, karakterističnih za strupe viperidnih kač: MP, SP, LAAO, PLTC (oziroma snaclec), disintegrinov, CRISP in sPLA₂ [12, 127]. Med peptidnimi komponentami smo v gadovem strupu identificirali natriuretične peptide in tripeptidne inhibitorje MP. Najbolj bogato zastopane proteinske družine v obeh strupih so MP, SP, sPLA₂ in CRISP. V gadovem strupu smo identificirali P-III MP, podobne modrasovima hemoraginoma VaH3 in VaH4 ter aktivatorju FX, VAFXA-II [76, 77, 78]. V gadovem strupu so tako kot v modrasovem prisotni tudi P-I MP, medtem ko predstavnikov P-II MP nismo našli. Slednje se v strupu modrasa običajno posttranslacijsko proteolitično procesirajo na P-I MP in disintegrin [76]. Verjetno se podobno dogaja tudi v strupu gada. SP so v gadovem tako kot v modrasovem strupu prisotni v večjem deležu glede na ostale komponente. Njihovi biološki učinki so po večini povezani s strjevanjem krvi, kar v veliki meri določa toksičnost strupa. V strupu gada smo identificirali eno bazično antikoagulantno sPLA₂ brez nevrotoksične aktivnosti, ne pa tudi nevrotoksičnih sPLA₂, podobnih Atx. Poleg te pa še protein, podoben AtnL iz modrasovega strupa. AtnL je miotoksičen in encimsko neaktiven homolog sPLA₂.

Imunološka primerjava strupov s štirimi različnimi protistrupi, proti celotnemu modrasovemu strupu (anti-*Vaa*), proti hemoragičnim komponentam (anti-H), proti nevrotoksičnemu AtxA (anti-Atx) in proti netoksičnemu AtnL₂ (anti-AtnL₂), je potrdila opažene razlike in podobnosti med strupoma, ki smo jih detektirali s proteomskim pristopom. Anti-*Vaa* in anti-H sta dobro prepoznala visokomolekulske komponente, v glavnem MP. Po pričakovanju je anti-AtxA prepoznal komponente samo v modrasovem strupu. Nasprotno je anti-AtnL₂ pokazal prisotnost AtnL₂ oziroma njegovih ortologov v obeh strupih, kljub temu da jih s proteomsko analizo v gadovem strupu nismo identificirali.

Moj prispevek v tem delu je bila analiza strupov z dvodimenzionalna poliakriamidna gelska elektroforeza (2DE) in s pomočjo RP-HPLC, priprava ločenih frakcij gadovega strupa za analizo z MS in izvedba kliničnih testov strjevanja krvi. Sodelovala sem tudi pri pisanju članka in urejanju slik.



Venomics of *Vipera berus berus* to explain differences in pathology elicited by *Vipera ammodytes ammodytes* envenomation: Therapeutic implications



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ABSTRACT

Vipera berus berus (Vbb) is the most widely distributed and *Vipera ammodytes ammodytes* (Vaa) the most venomous viper in Europe. In particular areas of the Old continent their toxic bites constitute a considerable public health problem. To make the current envenomation therapy more effective we have analysed the proteome of Vbb venom and compared it with that of Vaa. We found the proteome of Vbb to be much less complex and to contain smaller levels of particularly snakeheads and sPLA₂s. Snakeheads are probably responsible for thrombocytopenia. The neurotoxic sPLA₂s, ammodytoxins, are responsible for the most specific feature of the Vaa venom poisoning — induction of signs of neurotoxicity in patients. These molecules were not found in Vbb venom. Both venoms induce haemorrhage and coagulopathy in man. As Vaa and Vbb venoms possess homologous P-III snake venom metalloproteinases, the main haemorrhagic factors, the severity of the haemorrhage is dictated by concentration and specific activity of these molecules. The much greater anticoagulant effect of Vaa venom than that of Vbb venom lies in its higher extrinsic pathway coagulation factor-proteolytic activity and content of ammodytoxins which block the prothrombinase complex formation.

Biological significance: Envenomations by venomous snakes constitute a considerable public health problem worldwide, and also in Europe. In the submitted work we analysed the venom proteome of *Vipera berus berus* (Vbb), the most widely distributed venomous snake in Europe and compared it with the venom proteome of the most venomous viper in Europe, *Vipera ammodytes ammodytes* (Vaa). We have offered a possible explanation, at the molecular level, for the differences in clinical pictures inflicted by the Vbb and Vaa venoms. We have provided an explanation for the effectiveness of treatment of Vbb envenomation by Vaa antiserum and explained why full protection of Vaa venom poisoning by Vbb antiserum should not be always expected, especially not in cases of severe poisoning. The latter makes a strong case for Vaa antiserum production as we are faced with its shortage due to ceasing of production of two most frequently used products.

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1. Introduction

Venomous snakes make use of a complex cocktail of proteins and peptides in their venoms as a deadly weapon for hunting and defence. Of the six European endemic species of vipers, *Vipera latasti*, *Vipera xanthina*, *Vipera ursinii*, *Vipera aspis*, *Vipera berus* and *Vipera ammodytes*,

the last three are those, according to the WHO Guidelines 2010, whose bite is of the highest medical importance. The average number of snakebite cases per year between 1970 and 2010 in Europe has been estimated to be 7992 (~1 per 100,000 inhabitants), of which approximately 15% were considered severe and 4 were fatal [1]. We focused our study on the two subspecies that are most relevant from the medical point of view, *Vipera berus berus* (Vbb) and *Vipera ammodytes ammodytes* (Vaa). The former, known as the adder, or common viper, is the most widely distributed viper in Europe, causing more bites than any of its congeners [1,2]. The latter, also called the nose-horned viper, the Western sand viper, the long-nosed viper or the rhinoceros

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viper, is the most venomous European snake, responsible for the majority of severe poisoning cases, especially in the south/eastern parts of Europe where it is the most common venomous snake and whose encounter with people is relatively frequent [3–5].

The principal local effects of Vbb venom poisoning are haemorrhage, oedema, myonecrosis, bruising and pain [2,6–8], and hypotension is the most important sign of systemic envenomation [8–11]. This is reflected in gastrointestinal problems, with recurrent vomiting, nausea, circulatory instability and haematological disturbances. A patient may feel faint, and children in particular may become drowsy or semiconscious [2,12]. Other systemic effects that can appear include abdominal colic, incontinence of urine and faeces, sweating, vasoconstriction, tachycardia, and angio-oedema of the face, lips, gums, tongue, throat, and epiglottis, urticaria and bronchospasm [2,10,11]. There is some evidence that the venom contains cardiotoxic components that can cause electrocardiographic T wave inversion or flattening, ST elevation, second degree heart block and brady/tachyarrhythmias, atrial fibrillation, and myocardial infarction [10,13,14]. Laboratory tests have frequently revealed thrombocytopenia, neutrophilia, elevated serum creatine phosphokinase, elevated creatinine and metabolic acidosis [2,7,10,14]. Haemoglobin may remain high at first, but anaemia may occur later [2,10,14]. Reports of neurotoxic effects [8,15–17], systemic haemorrhage and coagulopathy at Vbb envenomation are rare [2,11].

The most comprehensive description of clinical manifestations caused by Vaa venom in man has been presented by Lukšić et al. [4] and Karabučić et al. [5] who have reviewed several hundreds of snakebite cases in southern Croatia. The main clinical signs that appear following the venomous bite of Vaa result from the hematotoxic and neurotoxic activity of the venom. All the envenomed persons displayed extensive swelling and oedema, over 90% had discoloration of skin and ecchymosis. Cranial nerve paresis or paralysis was the most common subsequent general complication (16%) most often reflected in ptosis, ophthalmoplegia, dysphonia, dysphagia, swelling problems and neuromuscular weakness. A relatively high percentage (5.9%) of the patients suffered central nervous system (CNS) depression, some of them (1.1%) without exhibiting shock, hypotension, or cranial nerve paresis or paralysis. This indicates that Vaa venom does not only have neurotoxic activity on cranial nerves but can also exert a direct toxic action on the CNS.

Proteomic and transcriptomic studies in recent years have provided a comprehensive insight into the complexity and functioning of many snake venoms. State-of-the-art analytical approaches have greatly accelerated the elaboration of more effective treatments of snake venom poisoning [18]. The risk of severe envenoming snakebites in Europe is relatively small. Nevertheless, in particular areas, for example in Mediterranean countries, snake venom poisoning represents considerable public health issue arguing for further improvements [4,5]. Venom proteomes of two European viper subspecies, Vaa and *Vipera ammodytes meridionalis* (Vam) have been reported so far [19]. The venom proteome of Vbb is presented in this work.

The registered clinical symptoms and the signs of envenomation by Vaa venom differ in several aspects from those of Vbb venom. For example, systemic haemorrhage and coagulopathy are usually much more severe in the case of the former. The most striking difference in pathological action on man is, however, in eliciting neurotoxic effects. These are very rarely observed in the case of Vbb venom bites but frequently in that of Vaa and constitute a medical emergency, since they can progress from ptosis to intense muscular weakness that can persist for several hours [4–10]. By analysing the Vbb venom proteome we proposed an explanation, at the molecular level, of the differences in clinical pictures inflicted by the Vbb and Vaa venoms. We have provided an explanation for the effectiveness of treatment of Vbb envenomation by Vaa antiserum and explained why full protection of Vaa venom poisoning by Vbb antiserum should not be always expected, especially not in cases of severe poisoning. The latter makes a strong case for Vaa antiserum production as we are faced with its shortage due to ceasing

of production, in Croatia and in France (both in 2015), of two most frequently used products.

2. Material and methods

2.1. Venoms

Commercial freeze-dried Vbb venom, obtained from Russia (Serpentarium of the Central Trade Base 'Zoo-obyedinenie' Khimki, Moscow District), was the generous gift of Dr. Jüri Siiger from the National Institute of Chemical Physics and Biophysics, Tallinn, Estonia. Crude Vaa venom was obtained from the Institute of Immunology, Zagreb, Croatia. The venom was lyophilized and stored at -20°C .

2.2. RP-HPLC fractionation

The composition of Vbb and Vaa venoms was analysed by HPLC (Flexar FX10, PerkinElmer, USA). 25 μL of venom (5 mg/mL) was dissolved in 625 μL 0.1% (v/v) TFA (solvent A) and applied on a reversed phase (RP) column PLRP-S (300 Å, 8 μm ; 4.6 $\times$ 150 mm; Agilent Technologies). The column was eluted with an increasing concentration of solvent B (90% (v/v) ACN and 0.1% (v/v) TFA) at 1 mL/min using the following gradient: 0–45% (v/v) B in 5 min, 45–100% (v/v) B in 45 min. Fractions were collected manually and dried in a SpeedVac vacuum concentrator (Savant, USA). Fractions were analysed using SDS-PAGE as described below. Fractions 1 to 6, containing components of lower molecular mass not visible on 12% (w/v) polyacrylamide gel, were pooled and re-chromatographed on an RP-HPLC C18 column (80 Å, 5 μm ; 4.6 $\times$ 30 mm; PerkinElmer, USA). Components were eluted at 1 mL/min using the same solvents as for PLRP-S analysis, starting at 0% solvent B and increasing with solvent B to 100% (v/v) in 58 min. The isolated fractions were submitted to the N-terminal amino acid sequencing.

2.3. N-terminal amino acid sequencing

N-terminal amino acid sequences of peptides and proteins were determined by Edman degradation on a Procise 492A Automated Sequencing System (Applied Biosystems, USA).

2.4. 1-D SDS-PAGE

Fractions of Vbb and Vaa venoms obtained by RP-HPLC separation and the products of cleavage of fibrinogen by the two venoms were analysed using 12% (w/v) and 12.5% (w/v) polyacrylamide gels in the presence of SDS (SDS-PAGE) under non-reducing conditions [20]. Proteins in gels were visualized by silver staining using a modified method of Morrissey [21] where the protein fixation was accomplished by the mixture of 30% (v/v) ethanol and 10% (v/v) acetic acid. The glutaraldehyde fixation step was omitted. In the case of Vbb venom, protein spots were excised from the gel and processed for assessment by mass spectrometry (MS). Molecular mass standards were from Fermentas (Lithuania).

2.5. 2-DE

2.5.1. First dimension – IEF

Freeze-dried Vaa or Vbb venom was dissolved in double distilled water giving final total protein concentrations of 5 mg/mL. 4 μL of the solution was added to 121 μL of rehydration buffer containing 7 M urea, 2 M thiourea, 30 mM Tris, 2.5% (w/v) CHAPS detergent, 0.25% (w/v) ASB-14 detergent, 1.5% (v/v) DeStreak reagent (GE Healthcare, Sweden), 1% (v/v) IPG buffer, pH 3–11 nonlinear (NL), (GE Healthcare, Sweden) and 0.002% (w/v) bromophenol blue. The sample was loaded on a 7 cm IPG strip (Immobiline DryStrip) pH 3–11 NL (GE Healthcare, Sweden). The strip was rehydrated overnight at room temperature.

Proteins were submitted to IEF on an IPGphor electrophoresis apparatus (GE Healthcare, Sweden) with the following running conditions: step 1: 300 V for 45 min, step 2: 1000 V for 30 min, step 3: 5000 V for 80 min and step 4: 5000 V until 6000 Vh per strip was reached.

2.5.2. Second dimension – SDS-PAGE

Following the IEF, the IPG strip was incubated at room temperature in 65 mM DTT in equilibration buffer containing 6 M urea, 125 mM Tris/HCl (pH 7.5), 4% (w/v) SDS and 0.002% (w/v) bromophenol blue for 15 min with sporadic shaking. After reduction, the strip was alkylated in equilibration buffer containing 135 mM iodoacetamide under the same conditions. The strip was then washed in the SDS-PAGE running buffer (0.3% (w/v) Tris, 1.44% (w/v) glycine and 0.1% (w/v) SDS), placed onto 12.5% (w/v) polyacrylamide gel and covered with 0.5% (w/v) agarose solution containing 0.02% (w/v) bromophenol blue and 1% (w/v) SDS. The SDS-PAGE was run at 5 mA per gel with gradual increase of current up to 15 mA per gel as described [22]. Gels were stained with colloidal silver as described above and imaged with optical Image Scanner using LabScan 5 software (GE Healthcare Life Sciences, USA). Gel scans were analysed using Image Master 2D Platinum 6.0 software (GE Healthcare Life Sciences, USA).

2.6. Mass spectrometry

In-gel tryptic digestion of Vbb venom proteins, their extraction from the gel and analysis on an IT mass spectrometer 1200 series HPLC-Chip-MS/MSD Trap XCT Ultra (Agilent Technologies, Germany) were carried out as described [22]. Proteins were identified using Spectrum Mill software Rev A.03.03.084 (Agilent Technologies, USA) against 'Snakes' (taxid 8750; 15,231 entries) protein database extracted from the non-redundant NCBI (National Center for Biotechnology Information) protein databank in May 2014, and supplemented with translated nucleotide sequences from Vaa venom deposited in NCBI GenBank in 2015 (KU249650–KU249656, KT148817–KT148834). The following search parameters were used: parent ion error tolerance of 2.5 Da, fragment error tolerance of 0.75 Da, maximum of 2 enzyme missed cleavages, peptide charges +2 and +3, methionine oxidation as variable modification and carboxamidomethyl cysteine as fixed modification. The results were validated using Scaffold software (version 2, Proteome Software, Inc., USA) with the following parameters: protein confidence of 95% and one peptide per protein at 95% confidence. N-terminally blocked short peptides were analysed using ESI Q-TOF-MS/MS (Waters-Micromass, UK) as described [23].

2.7. Western blot analysis

20 µg of Vaa or Vbb raw venom was separated by 2-DE as described above. Proteins were transferred to an NC2 PVDF membrane (Serva, Germany) at room temperature and 200 mA per gel for 90 min in Towbin transfer buffer (25 mM Tris/HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol). Following transfer, the PVDF membrane was blocked for 1 h at room temperature in 1% (w/v) Western blot blocking solution (WBS) (Roche, Germany) in TBS (50 mM Tris/HCl, pH 7.5 and 150 mM NaCl) and then incubated overnight with either rabbit anti-raw Vaa venom serum (anti-Vaa), anti-haemorrhagic venom fraction serum (anti-H), anti-ammodytin A serum (anti-AtxA) and anti-ammodytin I₂ serum (anti-AtnI₂) at a dilution of 1:1,000,000 (anti-Vaa and anti-H) and 1:10,000 (anti-AtxA and anti-AtnI₂) in 0.5% (w/v) WBS in TBS at 4 °C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgGs (1:10,000 in 0.5% (w/v) WBS in TBS) for 45 min at the room temperature. Spots were visualized with the ECL Lumi-LightPLUS Western Blotting substrate (Roche, Germany) and exposure to Amersham Hyperfilm ECL (GE Healthcare, UK) following the manufacturer's instructions.

2.8. Fibrinolytic activity assay

Fibrinolytic activity of Vaa and of Vbb venom was determined as described [24]. Human fibrinogen and venoms were dissolved in TBS at a concentration of 5 mg/mL. Fibrinogen and venom were incubated at a ratio of 100:1 (w/w) at 37 °C in either the presence or absence of a protease inhibitor, either EGTA (2.5 mM) or Pefabloc (1 mM). At 5, 15 and 60 min after mixing fibrinogen and the venom, 10 µL aliquots of the reaction mixture were analysed by SDS-PAGE as described above. Gels were stained with PageBlue® according to the manufacturer's instructions (Thermo Fisher Scientific, USA). Gels were digitized with Image Scanner and LabScan 5 software (GE Healthcare Life Sciences, USA).

2.9. Blood coagulation assays

Disturbance of the coagulation system by Vbb and Vaa venoms was assayed using three clinical blood coagulation tests: i) prothrombin time (PT) – the time needed for blood to coagulate after initiation of the extrinsic pathway by addition of thromboplastin and Ca²⁺ to citrated blood; ii) activated partial thromboplastin time (aPTT) – the time needed for clot formation after initiation of the intrinsic pathway by adding Ca²⁺, phospholipids, and kaolin to a sample of citrated blood; and iii) thrombin time (TT) – the time needed for fibrin clot formation after the addition of α-thrombin to citrated blood. 5 µL of Vbb or Vaa venom solution (25 µg total proteins), or 5 µL of buffer (20 mM MES, 2 mM CaCl₂, pH 7.5) containing no venom as a control, was added to 45 µL of pooled plasma (Control Plasma N) (Dade Behring, Germany) and incubated for 1 min at 37 °C. In the case of the PT assay, the coagulation process was initiated by the addition of 100 µL of Thromborel S reagent (Dade Behring, Germany), a source of thromboplastin and Ca²⁺ ions, while, in the case of the TT assay, the coagulation process was initiated by the addition of α-thrombin in 100 µL of BC Thrombin reagent (Dade Behring, Germany). In the aPTT assay, the plasma was incubated with 50 µL of Pathromtin SL Reagent (Dade Behring, Germany) for 2 min at 37 °C, after which the coagulation was initiated by the addition of 50 µL of 25 mM CaCl₂. All times were measured on a Behring Coagulation Timer (BCT) System (Dade Behring, Germany) according to the respective assay protocol [25].

2.10. Platelet aggregation assay

Blood was donated by the experimenter in accordance with permission No. 53/08/11 of the National Medical Ethics Committee of the Republic of Slovenia. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) was prepared as described [26]. 250 µL of PRP was pre-incubated with and without 25 µg of Vbb or Vaa venom for 5 min at 37 °C. 25 µL of collagen (DiaMed, Switzerland), to a final concentration of 10 µg/mL, ristocetin (Chrono-Log Corporation, USA) to a final concentration of 1 mg/mL, or ADP (Chrono-Log Corporation, USA), to a final concentration of 10 µM, were added and the change in the optical density monitored for 5 min using a Model 700 Whole Blood/Optical Lumi-Aggregometer (Chrono-Log Corporation, USA). The PRP optical density read represented 100% while that of PPP represented the 0% value [25].

2.11. Twitch tension recording of mouse phrenic nerve-hemidiaphragm preparation

Muscle contraction was measured as described [27]. Briefly, the diaphragm muscle with the associated phrenic nerves was dissected out and kept in an oxygenated bath containing Krebs–Ringer solution. Each hemidiaphragm preparation was pinned on its lateral side to a silicone-coated glass chamber. The hemidiaphragm medial side with tendon was attached to a silk thread via a stainless-steel hook to an isometric mechano-electrical transducer, Grass Force-displacement

transducer FT03 (Grass Instruments, USA). Nerve-evoked contraction, direct muscle stimulation and directly/indirectly elicited tetanic muscle contractions of the isolated mouse hemidiaphragm were followed in the presence of raw Vbb venom at room temperature. The experimental protocol was as follows: indirect muscle stimulation (N) 10 min, direct muscle stimulation (D) 3 min, tetanic indirect muscle stimulation (Tn), pause 3 min, tetanic direct muscle stimulation (Td), pause 6 min, N 1 min, D 1 min, N – 10 min control recording, addition of Vbb venom to a final concentration of 125 µg/mL, N 150 min, D 5 min, Tn, pause 3 min, Td, pause 3 min, 4 times washing with Krebs–Ringer solution, N 15 min, D 5 min, Tn, pause 3 min, Td, pause 3 min.

3. Results

3.1. Comparative functional analysis of Vbb and Vaa venoms

3.1.1. Electrophysiology on mouse phrenic nerve-hemidiaphragm preparation

Under controlled experimental conditions, no change in amplitude of twitch and tetanic contractions was observed during the 90 min exposure. The muscle tone remained at the baseline throughout the entire duration of the experiment (Fig. 1A). 125 µg/mL of the Vbb venom, however, completely blocked nerve-evoked as well as directly elicited single twitch responses and indirectly and directly evoked tetanic contractions (Fig. 1B). At room temperature, the venom produced a 50% block in 66.5 ± 6.6 min and a complete block of the nerve-evoked twitch responses of the muscle in 142 ± 7.1 min ($n = 3$). Nerve-evoked and directly evoked single twitch contractions and tetanic contractions did not recover after 20 min of extensive washing of the preparation with venom-free Krebs–Ringer solution. Based on the increase of basal tension and the complete block of both directly evoked muscle twitch and tetanic contractions, the effect of Vbb venom on the isolated mouse neuro-muscular (NM) preparation is concluded to be predominantly myotoxic [28].

3.1.2. Fibrinogenolytic activity

Both viperid venoms were able to cleave fibrinogen – the last protein in the coagulation cascade (Fig. 2). Degradation by Vaa venom was more intensive. This venom was also less specific, cleaving effectively α - and β -chains of the substrate, while Vbb venom apparently contained only α -fibrinogenases. Experiments in the presence of specific inhibitors of SVMPS or SVSPs, i.e. EGTA or Pefablock (PB), revealed that, in both venoms, fibrinogenolytic activity was mainly associated with SVMPS.

3.1.3. Effect on blood coagulation

Effects of Vbb and Vaa venoms on blood coagulation were compared by measuring PT (influence on tissue factor or extrinsic pathway), aPTT (influence on contact activation or intrinsic pathway) and TT (influence on common pathway). The most significant difference between the two venoms was in the measured PT, which was about three-fold longer in the presence of Vaa venom than of the Vbb venom. According to the control experiment Vaa venom prolonged PT by almost 170%, thus acting anticoagulant, while Vbb venom shortened it by about 20%, thus displaying a procoagulant effect (Fig. 3). Both venoms shortened the aPTT, Vaa venom by 38% and Vbb venom by 56%, while their effect on the TT was similar, i.e. a prolongation of the platelet coagulation time by about 10% (Fig. 3). From clinical reports, both venoms induce bleeding *in vivo* that is typically more severe in the case of Vaa venom poisoning. It was also reported that bleeding diathesis is not typical of Vbb venom poisoning [2,11]. The *in vitro* blood coagulation results here confirmed these observations by establishing the anticoagulant nature of Vaa venom and a balance between anti- and procoagulant effects in the case of Vbb venom poisoning.

3.1.4. Effect on platelet aggregation/agglutination

Platelet aggregation, induced either by ADP or collagen, and platelet agglutination, induced by ristocetin, was effectively inhibited in the presence of both viper venoms (Fig. 4). Irrespectively of the mode of induction, the inhibition of the platelet aggregation/agglutination process

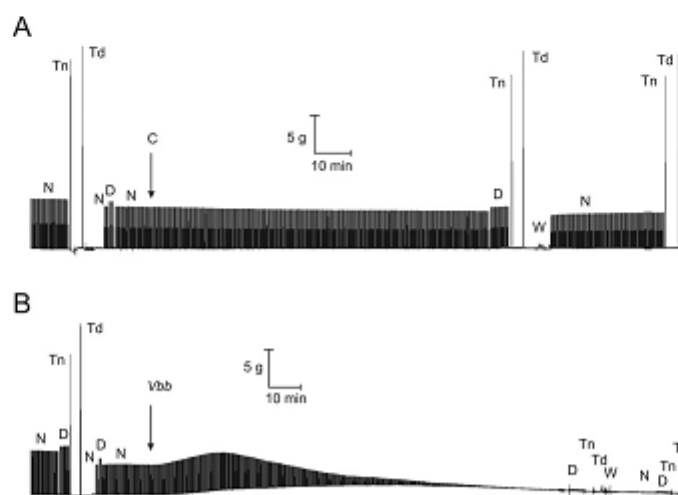


Fig. 1. The effect of Vbb venom on the mouse NM preparation. Mouse phrenic nerve-hemidiaphragm preparation was electro-stimulated indirectly or directly, and the muscle twitching was recorded. (A) Response of the muscle in control experiment (C + arrow indicate the addition point of the buffer containing no venom). (B) The effect of 125 µg/mL of the venom (Vbb + arrow indicate the addition point) on the nerve evoked "N" and directly elicited "D" muscle twitch amplitude, nerve-evoked tetanic contraction "Tn" and directly elicited tetanic contraction "Td". W: wash with Krebs–Ringer solution. Measurements were performed at the room temperature.

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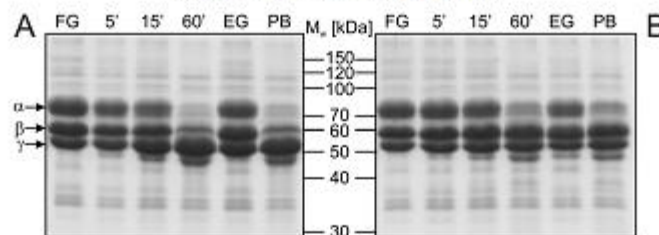


Fig. 2. Fibrinogenolytic activity of the viper venoms. Human fibrinogen was incubated with Vaa venom (A) or Vbb venom (B) at a 100:1 mass ratio at 37 °C for the indicated periods of time. The FG lanes contain control where the fibrinogen was incubated for 60 min in the absence of venom. Samples were incubated for 60 min at 37 °C with the respective venom also in the presence of EGTA (EG), the inhibitor of SVMs, or Pefabloc (PB), the inhibitor of SVSPs. SDS-PAGE analyses were performed under reducing conditions. The gels were stained with PageBlue®. Positions of fibrinogen α , β and γ chain and molecular mass standards are indicated.

was always almost complete in the presence of Vaa venom but not of Vbb venom. The latter powerfully inhibited the collagen-induced platelet aggregation while the ADP- and ristocetin-induced processes were inhibited by only about 50%.

3.2. Proteomic analysis of Vbb venom and its comparison with Vaa venom

3.2.1. Two-dimensional gel electrophoresis

Comparative 2-DE analysis of crude Vbb and Vaa venoms includes proteins with molecular masses of 10 to 150 kDa, having isoelectric points (pIs) in the pH range 3–11. A distinctive difference between the venoms is that Vbb venom displayed far fewer protein spots than Vaa venom (Fig. 5). The former was resolved into 160 distinct spots and the latter into 260. The distribution of spots on the gel however was similar for both venoms. The relative intensities of the corresponding spots in the two venoms mostly differed (Fig. 5). Some proteins were highly expressed in Vbb venom and some others in Vaa venom. In both venoms, the components are spread similarly between the acidic and neutral areas on the 2-DE gel. Characteristically, Vaa venom contains proteins that are much more basic than those in Vbb venom (Fig. 5). The former is also much richer in components with molecular masses lower than 20 kDa and higher than 60 kDa.

3.2.2. Structural proteomics

To identify proteins in Vbb venom structurally, the crude venom was first resolved into 14 fractions by RP-HPLC, using the PLRP-S column (Fig. 6B). Fractions 7 to 14 were further analysed by SDS-PAGE to

separate proteins by mass in the range of 15 to 150 kDa. Under non-reducing conditions, 30 discrete protein bands were detected on the gel by silver staining (Fig. 7). The bands were excised, proteins in-gel digested, extracted and then analysed by MS. 31 different proteins were identified (Table 1) representing all major protein families of viperid venoms: SVSPs, SVMs, sPLA₂s, CRISPs, LAAOs, snake venom metalloproteases and disintegrins. Interestingly, identified for the first time on the protein level in snake venoms, one of the proteins is a member of the aspartic protease family (AspP) [29]. Drawing a parallel with the Vaa venom 2-DE pattern, spots in the gel above 60 kDa represent various isoforms of LAAOs, P-III SVMs and snake venom metalloproteases, spots of intermediate mass (20 to 50 kDa) represent SVSPs, P-I SVMs and CRISPs, and spots below 20 kDa reflect the abundance of sPLA₂ molecules and disintegrins. Although fractions PRLP-S 1 to 6 were analysed under the same conditions by SDS-PAGE, no protein band was seen by silver-staining, suggesting the presence of only low molecular mass components in these RP-HPLC fractions. They were pooled and re-chromatographed on a RP-C18 HPLC column into 12 peaks (Fig. 6C). Using either Edman degradation or ESI Q-TOF-MS/MS we identified in these peaks inhibitors of SVMs, natriuretic peptides, a Kunitz-type protease inhibitor (inhibitor of SVSPs), an sPLA₂-like myotoxic amodylin L (AtrnL) and a covalently heterodimeric disintegrin VB7 (Table 2). Interestingly, also N-terminal peptides of serine proteases orthologous to VaaSP1 [30], VaaSPH-1, VaaSP-2 and VaaSP-4 were identified. Probably degradation products of these proteins were detected since, according to their molecular masses, they should be visible on SDS-PAGE analysis but were not. Volume (area $\times$ intensity) of the SDS-PAGE protein

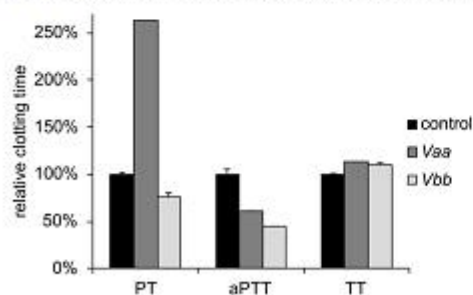


Fig. 3. Effect of the viper venoms on blood coagulation. Influence of the Vbb and Vaa venom on blood coagulation was analysed using three clinical blood coagulation assays: prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT). In controls, clotting times were measured in the absence of venoms and these values were taken as 100%. Measurements were performed at 37 °C on a Behring Coagulation Timer System using the respective standard assay protocol. Results are expressed relative to control values and displayed as means $\pm$ SD of duplicate measurements.

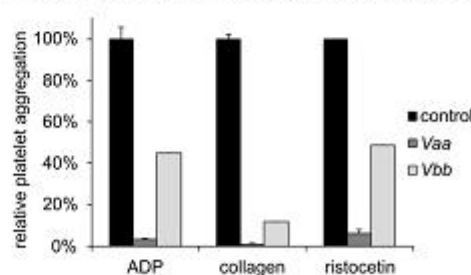


Fig. 4. Effect of the viper venoms on aggregation or agglutination of platelets. Influence of the Vbb or Vaa venom on platelet aggregation/agglutination was assessed at 37 °C by measuring a change in optical density of the platelet solution on a Model 700 Whole Blood/Optical lumi-Aggregometer. Aggregation of blood platelets was induced with the addition of either ADP or collagen, while their agglutination was induced with the addition of ristocetin. Control experiments, in which the aggregation or agglutination of platelets was measured in the absence of venoms, gave values that were taken as 100%. All other results are expressed relative to control values and displayed as means $\pm$ SD of duplicate measurements.

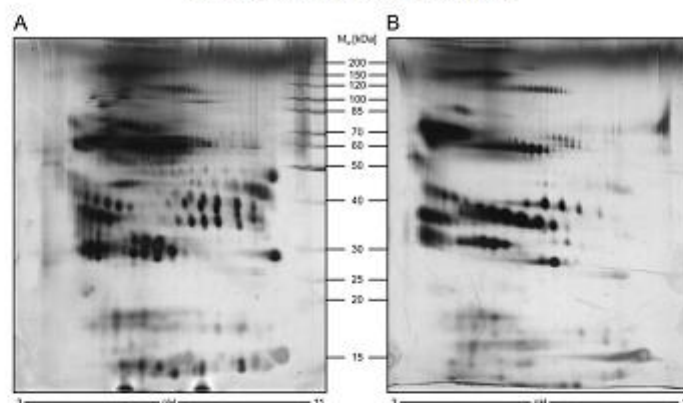


Fig. 5. 2-DE comparison of the viper venoms. 20 µg of Vaa venom (A) and the same amount of Vbb venom (B) were separated on the 7 cm IEF strips, pH 3–11, in the first, and on 12.5% (w/v) polyacrylamide gel under reducing conditions, in the second dimension. Gels were silver stained.

bands and area of peaks eluting at the HPLC analysis of the Vbb venom were used to estimate the quantity of proteins/peptides belonging to different polypeptide families in the venom. In Fig. 8, the pie chart is presented, which shows the ratios between the major polypeptide families in the Vbb venom.

3.3. Immunological comparison of the Vbb and Vaa venoms

The two venoms were first analysed using 2-DE; the proteins were then electroblotted from the gel onto a PVDF membrane and incubated with rabbit antisera against the following antigens: raw Vaa venom, the most neurotoxic venom component AtxA, the non-toxic sPLA₂ enzyme Atnl₂ and the total haemorrhagic fraction of Vaa venom. Anti-AtxA was shown to react exclusively with ammodytoxins (Atxs), presynaptically neurotoxic sPLA₂ isoforms, but not also with paralogous ammodytoxins (Atns), non-toxic sPLA₂s or myotoxic sPLA₂-like proteins, from Vaa venom [31]. Similarly, anti-Atnl₂ recognized Atns but not Atxs. Anti-Vaa recognized particularly strongly Vaa venom proteins with masses above 40 kDa. Anti-Vaa also recognized proteins in heterologous Vbb venom with comparable strength. Interestingly, the number of venom components in Vbb that reacted with anti-Vaa was larger than those in Vaa (Fig. 9 – top half). A similar result was obtained with anti-H (Fig. 9 – bottom half). Anti-AtxA recognized antigens only in Vaa venom (Fig. 10 – top half) while the orthologues of Atnl₂ were detected also in Vbb venom (Fig. 10 – bottom half).

4. Discussion

4.1. The Vbb venom proteome is less complex than that of Vaa

The venom proteome of Vbb has been analysed and compared to available proteomic data for Vaa venom from the literature. Under identical conditions, raw Vbb venom was separated by 2-DE into 160 distinct spots and Vaa venom into 260 (Fig. 5). Consistent with the results of 2-DE analysis, Vaa venom was resolved using the PLRP-S RP-HPLC column into almost twice as many peaks as Vbb venom under identical conditions (Fig. 6B). The venom of Vaa is thus more complex than that of Vbb. Immuno-reactivity patterns demonstrated that all the major protein groups present in Vaa venom are present in Vbb venom also (Figs. 8 and 9). The antibodies directed against whole Vaa venom (anti-Vaa) and those directed only against its haemorrhagic fraction (anti-H) recognized similar populations of molecules in heterologous venom (Fig. 9). A striking immunological difference between

the two venoms was however noted when immuno-staining was carried out with anti-AtxA serum (Fig. 10 – top half). While a strong signal was obtained in the case of the Vaa venom analysis, it was completely absent from the analysis of Vbb venom. This result suggests that there is no Atx-like sPLA₂ molecule in Vbb venom.

4.2. Protein families present in Vbb venom

4.2.1. Snake venom serine proteases (SVSPs)

SVSPs mostly affect hemostasis in mammals, by influencing blood coagulation, fibrinolysis, platelet aggregation and vasoconstriction [32]. They belong to the subfamily S1A chymotrypsin-like proteases and show high macromolecular trypsin-like substrate specificity [33]. Most SVSPs are N- or O-glycosylated, but to different extents. As reported for Vaa venom [19], diverse SVSPs are abundant in Vbb venom also (Table 1). Vbb SVSPs, with apparent molecular masses of 25, 35, 40 and 55 kDa, were identified in PLRP-S fractions 8 to 14 by similarity to SVSPs from related snakes, Vaa, *Macrovipera lebetina* (M. lebetina), *Crotalus atrox* (C. atrox), *Agkistrodon contortrix contortrix* (A. c. contortrix) and *Trimeresurus stejnegeri* (T. stejnegeri). These Vbb SVSPs may thus exert thrombin-like, fibrinogenolytic and kallikrein-like activities [34–38]. However, as demonstrated in Fig. 2, the fibrinogenolytic activity of the Vbb and Vaa venoms is mainly caused by their SVMPs. A protein, probably a degradation product originating from VaSP1 [30], VaaSPH-1, VaaSP-2 or VaaSP-4-like Vbb molecule, was found in RP-C18 fraction 12 (Table 2).

4.2.2. Snake venom metalloproteases (SVMPs)

Most of the haemorrhagic activity of Vaa venom originates from its P-III SVMPs [39]. Antibodies directed against the haemorrhagic fraction of Vaa venom (anti-H) reacted strongly with molecules in Vbb venom with apparent molecular masses greater than 55 kDa (Fig. 9). SVMPs are synthesised as multi-domain enzymes, whose proteolytic activity depends on the presence of Zn²⁺ ions and is down regulated with citrate ions, acidic pH and competitive inhibitory tripeptides [40]. SVMPs are grouped structurally into three major classes: P-I, consisting of a protease domain (20–30 kDa), P-II, that consists of two domains, protease and a disintegrin (30–60 kDa), and the P-III class, that consists of three domains, protease, disintegrin and a cysteine-rich domain (60–100 kDa). SVMPs affect the hemostatic system in many different ways. They may degrade components of the capillary basement membrane, which results in haemorrhage, and prothrombin or factor X (FX), which leads to their depletion. They can act as prothrombin or

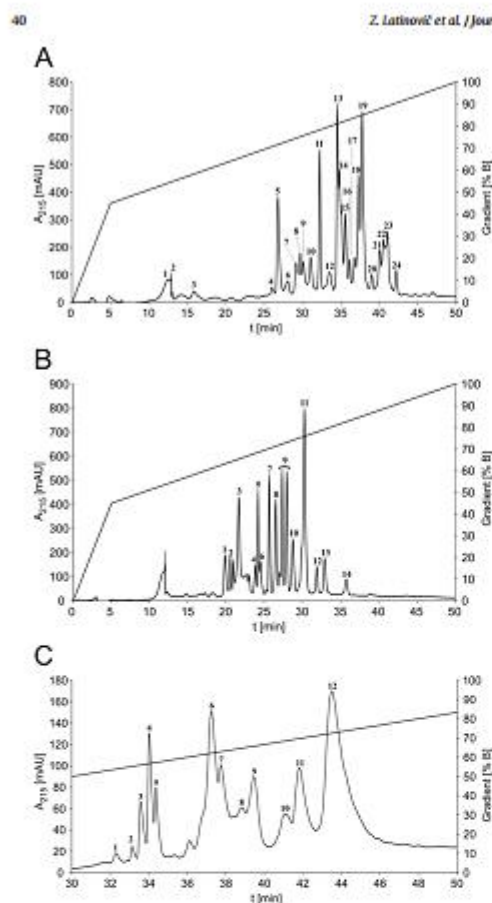


Fig. 6. Reversed phase HPLC analyses of the viper venoms. The RP-HPLC column PLRP-S (300 Å, 8 µm; 4.6 × 150 mm) was equilibrated in 0.1% (v/v) TFA (solvent A) and eluted with an indicated gradient of 90% (v/v) ACN in 0.1% (v/v) TFA (solvent B) at a flow rate of 1 ml/min. Chromatograms (A) and (B) display the analysis of the Vaa and Vbb venoms. (C) Vbb PLRP-S fractions 1 to 6 were pooled and further chromatographed on an RP-C18 column (80 Å, 5 µm; 4.6 × 30 mm) equilibrated in solvent A. At 1 ml/min, venom components were eluted using the indicated gradient of solvent B.

FX activators, inactivators of serpins, and as inhibitors of platelet aggregation [40,41]. Most SVMPs exhibit fibrinolytic activity. The presence of a haemorrhagic SVMP in Vbb venom was first reported in 1990 [42]. In our proteomic survey, we identified orthologues of the haemorrhagins Vah3 and Vah4, dimeric P-IIIc SVMPs from Vaa venom [25,43]. With apparent molecular masses above 100 kDa, these molecules were found in PLRP-S fractions 9, 13 and 14. Proteins in fraction PLRP-S 11 were identified as orthologues of the P-IIIb subclass SVMPs, characterized by a P-III SVMP with a covalently attached two-chain snakehead subunit. These are the coagulation FX activators VAFXA-II and VLFXA, the first present in Vaa [26] and the second in *M. lebetina* venom [44]. Also, Vbb orthologues of P-III SVMPs from *Echis carinatus* and *Bothrops erythromelas*, both known as prothrombin activators [45, 46], were found in PLRP-S fraction 11 (Table 1). SVMPs were also identified in the 33 kDa band of fraction PLRP-S 12. Two peptides identified in this fraction were sequentially identical to segments of VaaMPII-3 (KT148833), indicating the presence of unprocessed P-II SVMP in Vbb

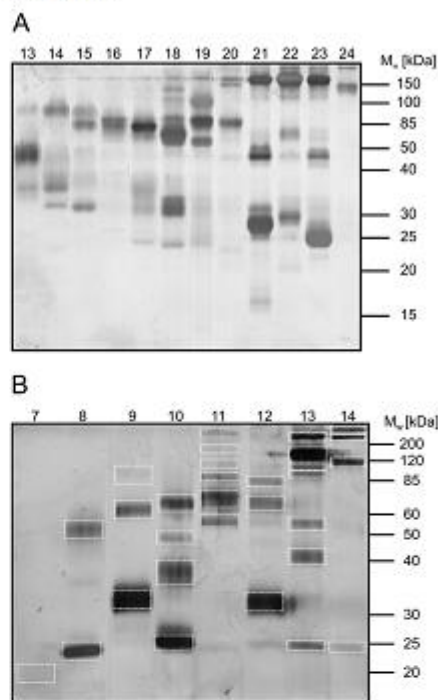


Fig. 7. SDS-PAGE analysis of the PLRP-S venom fractions. 12.5% (w/v) and 12% (w/v) polyacrylamide gels and non-reducing conditions were used to electrophorese Vaa (A) and Vbb (B) PLRP-S fractions. Gels were silver stained. Indicated Vbb protein spots (white rectangles) were excised from the gel and trypticized. Resulting peptide fragments were extracted from the gel and analysed by mass spectrometry.

venom. Two other peptides in this fraction originate from a disintegrin-like and cysteine-rich (DC) domain of a protein orthologous to Vah4-B [25]. Hemostatically active DC domains appear in venoms as products of proteolytic processing of the P-IIIb SVMPs (discussed under Disintegrins) [47]. Samel and Siigur [48] reported a Vbb SVMP of 38 kDa with FX activating activity (VbFXAE) and some of the peptides identified in this fraction may originate from this molecule.

For effective control of proteolytic activity, SVMP-rich snake venoms are usually rich in low molecular mass peptide inhibitors [49]. Such a molecule was identified in Vbb venom in the RP-C18 fraction 3 (Fig. 6C). Using ESI Q-TOF-MS/MS (Table 2), the inhibitor was identified as a tripeptide: pyroglutamate-lysine-tryptophan (pEKW). The same reversible inhibitor of SVMPs, a product of proteolytic processing of a longer translational product, has been described in other viper venoms [49].

4.2.3. Aspartic protease (AspP)

MS/MS analysis of the band with an apparent molecular mass of 40 kDa in the PLRP-S fraction 13 (Fig. 5) revealed the presence of two peptides characteristic of renin-like AspPs (Table 1). This is the first report of an AspP in snake venoms. At the transcriptional level, AspP was identified in the venom of the snake *Echis ocellatus* (*E. ocellatus*) [29]. The sequence of *E. ocellatus* AspP is highly similar to human and mouse renin and, their substrate specificity is presumably also similar. The main role of renin is regulation of systemic hypertension. It converts angiotensinogen, by proteolysis, to angiotensin I, which is further

Table 1
Assignment of the Vb venom high molecular mass protein fractions, separated in the first dimension on an RP-HPLC PLRP-S column (Fig. 6A) and then by SDS-PAGE (Fig. 7B), with the ESI ion trap mass spectrometer 1200 series HPLC-Chip-IC/MSD Trap XCT Ultra. Identification of proteins relies on the MS/MS-derived peptide sequences that are listed. Accession number and the main activity of the most similar identified proteins are quoted, and the protein family specified. Abbreviations: m, oxidized methionine; n, d, not determined.

HPLC fraction	SDS-PAGE [kDa]	Observed m/z	z	MS/MS-derived sequence	Related protein	NCBI accession number	S. Mill protein score	Coverage [%]	Main activity	Protein family
PLRP-S-7	30	976.15	2	NLFQFCNMNMHVCK	<i>sPLA₂</i> [Vbb]	P31854	38.02	18	Phospholipase [53]	<i>sPLA₂</i>
		468.80	2	VAAICFK						
PLRP-S-8	55	750.02	2	VVGZDECNNEHR	Centorrrobilin [A. c. centorrroix]	P62981	35.78	14	Thrombin-like [37]	SVSP
		1227.14	2	VFDYNDWQSIAGNTAATCP	Bilirubin [A. bilineatus]	Q9P5N3	17.76	5	Thrombin-like [35]	SVSP
		764.22	2	BGGZCNNEHR	Triflin [P. flavoviridis]	AAM45665	11.63	4	Ca ²⁺ -channel blocker [65]	CRISP
		583.66	2	NVDFSESPR	CRISP [Vbb]	CAP74089			n. d.	
					CRISP [V. nikolaki]	CAP74088	107.44	36	n. d.	CRISP
PLRP-S-8	25	775.71	2	CGENFYmSTSPmk						
		1386.46	2	DFVYQQASPANAVVGHVYQAVWYK						
		612.01	2	CNVDFSESPR						
		776.02	2	SGCAAVCPSEYK						
		1173.72	3	SGCAAVCPSEYKVFYVQVCPAGNMQCK						
		993.71	2	VYVYQVCPAGNMQCK						
		640.79	3	KPDQNEIDLHNSLR	TM-CRVP [P. microsquamatus]	P79845	110.01	23	n. d.	CRISP
		769.77	2	MEWYFEAANAER						
		659.53	2	RSVNPTASNMK						
		581.85	2	SVNFTASNMK						
		898.65	3	SVNFTASNMKMEWYFEAANAER						
		994.57	2	VYVYQVCPAGNMK						
		583.43	2	NVDFSESPR	Triflin [P. flavoviridis]	AAM45665	39.82	11	Ca ²⁺ -channel blocker [65]	CRISP
		1022.85	3	NVDFSESPRPEQNEIDLHNSLR	CRISP [Vbb]	CAP74089			n. d.	
PLRP-S-9	100	835.16	2	SCIMSTLSCKSR	VaH3 [Vaa]	KC007435	31.81	6	Haemorrhagin [43]	SVMP
		1246.51	2	QCISLFCASATVAQDACTQENR						
PLRP-S-9	65	583.43	2	NVDFSESPR	CRISP [Vbb]	CAP74089	79.76	21	n. d.	CRISP
		776.55	2	MEWYFEAANAER						
		993.73	2	VYVYQVCPAGNMQCK						
		580.66	2	SVNFTASNMK						
		1228.37	2	VFDYNDWQSIAGNTAATCP	VaaSP-2 [Vaa]	KT148825	18.95	8	n. d.	SVSP
		1227.85	2	VFDYNDWQSIAGNTAATCP	VLAF [M. leberina]	AAM96674	20.56	8	α -Fibrinogenase [38]	SVSP
PLRP-S-9	35	1227.14	2	VFDYNDWQSIAGNTAATCP	VLAF [M. leberina]	AAM96674	18.98	9	α -Fibrinogenase [38]	SVSP
PLRP-S-10	70	1097.07	2	IFEVNTMNMHPLNR	ACLD [A. c. latricinctus]	AAC18911	16.40	2	n. d.	SVMP
PLRP-S-10	50	1125.94	3	TSTHAPLSLSPSPVGVCR	VaaSP-3, VaaSP-5 [Vaa]	KT148826, KT148828	36.70	12	n. d.	SVSP
		560.17	2	AAYPWLLER	VLAF [M. leberina]	AAM96674	21.37	9	α -Fibrinogenase [38]	SVSP
		1227.72	2	VFDYNDWQSIAGNTAATCP	VaaSP-3, VaaSP-5 [Vaa]	KT148826, KT148828	37.04	10	n. d.	SVSP
PLRP-S-10	40	1125.98	3	TSTHAPLSLSPSPVGVCR						
		560.34	2	AAYPWLLER						
		829.45	3	VFDYNDWQSIAGNTAATCP	Stejselthase 2 [T. stejnegeri]	AAN52349	18.95	8	β -Fibrinogenase [36]	SVSP
		824.09	3	VFDYNDWQSIAGNTAATCP						
		1000.31	3	LNRPVKNASHPELSPSPVGVCR	KN6 [T. stejnegeri]	Q71QJ2	16.40	10	n. d.	SVSP
		1227.14	2	VFDYNDWQSIAGNTAATCP	VLAF [M. leberina]	AAM96674	14.04	9	Kallikrein-like [34]	SVSP
PLRP-S-10	25	769.97	2	MEWYFEAANAER	CRISP [Vbb]	CAP74089	32.92	10	n. d.	CRISP
		581.91	2	SVNFTASNMK						

(continued on next page)

Table 1 (continued)

HPLC fraction	SDS-PAGE [kDa]	Observed m/z	z	MS/MS-derived sequence	Related protein	NCBI accession number	S. Mill protein score	Coverage [%]	Main activity	Protein family
PURP-S-11	250, 150, 110	627,18 691,36	2	SAGQLYEESLR	LAAD [S. c. edwardsi]	ABC26996	36,48	2	n. d.	LAAD
			2	SAGQLYEESLRK						
		807,19	2	ATVAEDSCFDNQK	Berythrinase [B. erythronotus]	AAL47169	18,70	2	Prothrombin activator [46]	SVMP
PURP-S-11	90	534,75	2	KCVDNVTAY	Ecarrin [E. carinatus]	Q90495	24,08	3	Prothrombin activator [45]	SVMP
		847,35	3	LKPAECGCECCDQK						
		585,79	2	KPCAPQGVK	VLFXA, heavy chain [M. lebetina]	Q77046	34,88	4	FX activator [44]	SVMP
		594,28	3	LVCLDPSGKINPK						
		1057,91	2	AFCCPSCWSAYDQNCYK	VaaSP-3 [Vaa]	KU249654	23,53	12	n. d.	Snaclec
		854,42	2	TSADYVWGLWVQK	Lebecin subunit β [M. lebetina]	W5XQJ6	15,97	9	Anti-cancer, anti-integrin [68]	Snaclec
PURP-S-11	75	738,61	3	LHSVVECECECCDQCR	P-III SVMP [E. ocellatus]	CAJ01687	18,62	2	n. d.	SVMP
PURP-S-11	55	631,37	2	FWEDDGHGGR	VAA-LAAD I [Vaa]	P0D884	48,82	4	L-Amino acid oxidase [58]	LAAD
		464,18	3	KPWEDDGHGGR						
		502,66	2	VTVLEASER						
		627,18	2	SAGQLYEESLR	LAAD [S. c. edwardsi]	ABC26996	36,48	2	n. d.	LAAD
		691,36	2	SAGQLYEESLRK						
PURP-S-12	85	751,15	3	VFDYNDWIQSIAGNTAATCPP	VLAFF [M. lebetina]	AAM96674	21,37	8	α-Fibrinogenase [38]	SVSP
		1227,62	2	VFDYNDWIQSIAGNTAATCPP						
PURP-S-12	70	1012,56	2	FLQYNPKCMINKPLR	P-III SVMP [E. ocellatus]	CAJ01685	14,78	2	n. d.	SVMP
		677,99	2	NPQGLNKPLR	VaaMPB-3 [Vaa]	KT148833	22,11	2	n. d.	SVMP
		526,22	2	QCVDVNTAY	VaH3, VaH4-A, VaF1 [Vaa]	KC007435, AHB62069, AJC52543	13,92	1	Haemorrhagin, fibrinogenase [43,25,81]	SVMP
PURP-S-12	33	677,44	2	NPQGLNKPLR	VaaMPB-3 [Vaa]	KT148833	47,46	12	n. d.	SVMP
		884,06	2	YIELVIVADHSMFTK						
		662,66	3	LTPCAECGCECCDQCR	VaH4-B [Vaa]	Ref. [25]	35,29	5	Haemorrhagin [25]	SVMP [DC fragment]
		535,65	2	KCVDNVTAY						
PURP-S-13	250, 150, 110, 100	662,69	3	LTPCAECGCECCDQCR	VaH4-B [Vaa]	Ref. [25]	35,29	5	Haemorrhagin [25]	SVMP
		535,65	2	KCVDNVTAY						
PURP-S-13	55	677,14	2	NPQGLNKPLR	VaaMPB-3 [Vaa]	KT148833	19,73	2	n. d.	SVMP
		560,12	2	AAYPWLLER	VaaSP-3, VaaSP-5 [Vaa]	KT148826, KT148828	35,58	13	n. d.	SVSP
		751,25	3	TSTHAPLSLSPSPSPVCSVCR						
		1227,33	2	VFDYNDWIQSIAGNTAATCPP	VLAFF [M. lebetina]	AAM96674	21,64	8	α-Fibrinogenase [38]	SVSP
PURP-S-13	40	1016,39	2	DTQYYEISGTPAQFK	Remisin-like aspartic protease [E. ocellatus]	CAJ55260	31,28	6	n. d.	Aspartic protease
		567,78	2	QYIEFDR						
PURP-S-13	25	560,12	2	AAYPWLLER	VaaSP-3, VaaSP-5 [Vaa]	KT148826, KT148828	37,72	13	n. d.	SVSP
		751,25	3	TSTHAPLSLSPSPSPVCSVCR						
PURP-S-14	250, 200, 120	565,74	2	LCNEYGYCR	VaH3 [Vaa]	KC007435	176,82	30	Haemorrhagin [43]	SVMP
		889,01	3	NPCQYVTPEDENK						
		1246,47	2	QCISLFGASATVAQDAFCQFNK						
		514,94	2	KPCAPQGVK						
		578,81	2	KPCAPQGVK						
		663,15	3	LTPCAECGCECCDQCK						
		836,88	2	SCMSGTLSCFASIR						
		845,03	2	SCMSGTLSCFASIR						
		1442,54	2	TDVSPAVCCNVVELGECDCGSPR						
		446,86	2	VTDLFGK						
		618,73	2	VTDLFGKWR						
PURP-S-14	25	560,45	2	AAYPWLLER	VaaSP-3, VaaSP-5 [Vaa]	KT148826, KT148828	39,28	13	n. d.	SVSP
		751,26	3	TSTHAPLSLSPSPSPVCSVCR						
		1227,48	2	VFDYNDWIQSIAGNTAATCPP	VLAFF [M. lebetina]	AAM96674	9,63	8	α-Fibrinogenase [38]	SVSP

Table 2

Assignment of the Vbb venom components, separated on the RP-HPLC C-18 column (Fig. 6C), using Edman degradation and ESI Q-TOF-MS/MS. Identification of peptide/protein was made according to its N-terminal or MS/MS-derived sequence that is listed. NCBI accession number and the main activity of the most similar identified proteins along with the protein family are specified. Cys residues in peptides were not derivatized before Edman sequencing, so their occurrence in sequences is only inferred based on the absence of a signal and similarity to the known sequences (C). Abbreviation: n.d., not determined.

HPLC fraction	N-terminal sequencing or MS/MS	Protein	NCBI accession number	Main activity	Protein family
C18-3	pEKW	Tripeptide MP inhibitor precursor [Vaa]	KT148817, KT148818	SVMP inhibition [42]	–
C18-6	DNEPPK	Tripeptide MP inhibitor precursor [Vaa]	KT148818	n.d.	Natriuretic peptide
C18-7	DNEPPKKVPPNS(C)FGH80DR	Tripeptide MP inhibitor precursor [Vaa]	KT148818	n.d.	Natriuretic peptide
C18-8	DNEPP	Tripeptide MP inhibitor precursor [Vaa]	KT148818	n.d.	Natriuretic peptide
C18-9	DNEPP	Tripeptide MP inhibitor precursor [Vaa]	KT148818	n.d.	Natriuretic peptide
C18-10	SVIEFGKMIQ NSGNP(C)DPV ELLQNSGNP(C) RDRPKF(C)YLP	Ammodytin L [Vbb] V87A [Vbb] V87B [Vbb] VaaCh [Vaa]	CAE47258 P0C6A6 P0C6A7 AAP04485	Integrin $\alpha 5 \beta 1$ inhibition [72] SP inhibition [64]	Dimeric disintegrin Kunitz-type protease inhibitor
C18-11	SVIEFGKMIQ	Ammodytin L [Vbb]	CAE47258	n.d.	sPLA ₂
C18-12	VIGGDE(C)NIN	VasP1 VasSPH-1, VasSP-2, VasSP-4 [Vaa]	KT148824, KT148825, KT148827	Fibrin(ogen)ase [23]	SVSP

hydrolysed by angiotensin-converting enzyme (ACE) to vasoconstrictor angiotensin II [50]. By inducing local hypertension, possible roles of AspPs in snake venoms are promotion of tissue disruption caused by SVMPs and facilitation of the spread of venom through the victim's bloodstream.

4.2.4. Secreted phospholipases A₂ (sPLA₂)

Using specific anti-sPLA₂ antisera we showed that Vbb venom contains at least two Atx-like molecules, but no Atx-like sPLA₂ (Fig. 10). sPLA₂ molecules, characteristic of viperid venoms, belong structurally to the IIA group [51]. These are standard venom proteins exerting either digestive and/or toxic functions. Toxicity of sPLA₂ molecules can be manifested in many different ways, for example in neurotoxicity, myotoxicity, cardiotoxicity and anticoagulant activity [52]. Using tandem MS we identified two peptides in Vbb venom PLRP-S fraction 7 that are also present in the primary structure of VbbPLA₂, a basic anticoagulant sPLA₂ [53]. The primary structure of VbbPLA₂ is only 57% identical with the primary structure of the neurotoxic Atxs from Vaa venom [54] and, not surprisingly, these molecules are not immunologically cross-reactive (Fig. 10 – top half). The other type of sPLA₂ molecule

in Vbb venom was detected in the RP-HPLC fractions C18-10 and C18-11 (Fig. 6C, Table 2). Edman degradation showed that this molecule has the N-terminus identical to that of AtxL, a myotoxic sPLA₂-analogue from Vaa venom [55]. Suggestively, the myotoxicity of Vbb venom, indicated in an *ex vivo* twitch tension experiment on mouse NM preparation, is caused by this protein (Fig. 1). In accordance with our proteomic results, analysis of the sPLA₂ genes of Vbb from central France revealed the presence of genes encoding the VbbPLA₂, AtxL, AtxL₁ and AtxL₂ [56]. Interestingly, genes of the A and B subunits of vaspin, a heterodimeric neurotoxin similar to vipoxin [57], were also detected in this study. The authors speculated that vaspin is probably either expressed at a very low level or not at all in the studied Vbb venom, because no neurotoxic envenomation has ever been reported in the region where the snakes were captured. We have also not found neurotoxic sPLA₂s, either dimeric vaspin or monomeric Atxs, in Vbb venom investigated, in line with its non-neurotoxic nature.

4.2.5. L-Amino acid oxidases (LAOs)

The presence of LAOs in Vbb venom is indicated by its yellow colour. LAOs contain the prosthetic flavin adenine dinucleotide (FAD) group, the fully oxidized FAD group appearing yellow. LAOs have been found in PLRP-S fraction 11 (Table 1), in SDS-PAGE bands of apparent molecular mass 55, 110, 150 and 250 kDa. Under non-denaturing conditions, LAOs usually form homodimers, so LAOs identified in the 55 kDa band are probably monomers of the 110–250 kDa LAOs. In the 55 kDa band, peptides identical to the Vaa-LAO I from Vaa venom [58] or to the LAO from *Sistrurus catenatus edwardsii* (*S. c. edwardsii*) venom [59] have been identified. Only *S. c. edwardsii* LAO-peptides have been detected in the bands with higher masses. One of the LAO isoforms has already been isolated from Vbb venom and partially characterized [60]. The enzyme disturbed hemostasis by inhibiting ADP-induced platelet aggregation, and induced apoptosis in cultured HeLa and K562 cells. Both effects have been induced by hydrogen peroxide, a reaction product of oxidative deamination of L-amino acids by LAO.

4.2.6. Cysteine-rich secretory proteins (CRSPs)

CRSPs are 20 to 30 kDa proteins, containing 16 cysteines to form 8 disulfide bonds [61,62]. CRSPs are a frequent constituent of snake venoms and, not surprisingly, we found them also in Vbb venom (Table 1). CRSP peptides were most abundantly present in the fraction PLRP-S 8, in the 25 kDa protein band. Peptides were identified according to their identity with the respective parts of CRSPs from the venom of *V. berus* [63], and the *V. berus*-related vipers, *Vipera nikolskii* [63], *Protobothrops mucrosquamatus* (*P. mucrosquamatus*) (TM-CRVP) [64] and *P. flavoviridis* (triflin) [65]. A CRSP of apparent molecular mass 25 kDa was also identified in the fraction PLRP-S 10, with the two

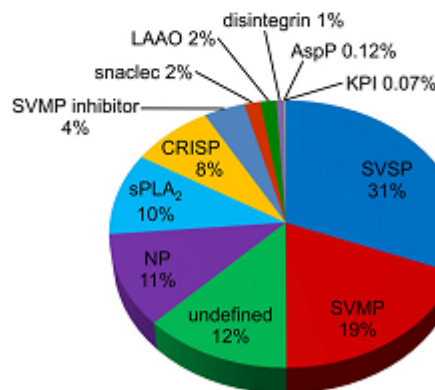


Fig. 8. Composition of Vbb venom according to protein and peptide families. Relative abundance of venom polypeptide families was calculated from the normalized volumes of corresponding SDS-PAGE protein bands and normalized areas of corresponding peaks eluting at the HPLC analysis of the Vbb venom. SVSP, snake venom serine protease; SVMP, snake venom metalloproteinase; LAAO, L-amino acid oxidase; sPLA₂, secreted phospholipase A₂; AspP, aspartic protease; CRISP, cysteine-rich secretory protein; snaclec, snake venom C-type lectin; NP, natriuretic peptide; KPI, Kunitz-type protease inhibitor.

peptides matching the structure of *V. berus* venom CRISP [63]. Interestingly, CRISPs were also detected in SDS-PAGE bands of 55 and 65 kDa (PLRP-5 fractions 8 and 9 in Figs. 6A and 7). Since, to our knowledge, snake venom CRISPs do not form dimers and are not glycosylated, we assume that on non-reducing SDS-PAGE they migrated in complex with an SVSP detected in the same band (Table 1). This suggests synergistic action of certain CRISPs and SVSPs [66]. Such a possibility is supported by the fact that, in spite of extensive testing, no pharmacological activity appears to have been detected in the case of the pure *Vaa* CRISP, the molecule 96% identical in its amino acid sequence (KT148819) to the *V. berus* CRISP (Žilajpah M, personal communication). Of all the snake venom CRISPs most similar to the *Vbb* venom CRISPs, the activity of only triffin (*P. flavoviridis* CRISP) has been determined [65]. Triffin inhibited the high K^+ -induced contraction of the rat-tail arterial smooth muscle, tentatively by blocking the conductance of the L-type Ca^{2+} channels.

4.2.7. Snaclecs

Snaclecs are characterized by a conserved region of 115–130 amino acid residues, homologous to the carbohydrate recognition domain (CRD). CRDs bind saccharides in a Ca^{2+} -dependent manner in C-type lectins while in snaclecs they lack the carbohydrate-binding activity [67]. In *Vbb* venom, snaclecs were only detected among the 90 kDa high molecular mass proteins in PLRP-5 fraction 11 (Table 1). Classic

snaclecs, disulfide-bonded $\alpha\beta$ -heterodimers of 13–15 kDa CRD-like subunits, have not been detected, which is very different from *Vaa* venom. In *Vaa* venom, snaclecs are abundantly present as proteins of 25–50 kDa [39]. These molecules are the major inducers of thrombocytopenia, which is clinically manifested as excessive platelet aggregation/agglutination. In contrast to our platelet aggregation/agglutination results obtained *in vitro* (Fig. 4), thrombocytopenia is relatively frequently encountered in patients poisoned by *Vbb* or by *Vaa* venom [4,10]. Since no agonists of platelet aggregation have been detected in the venoms, snaclecs may be responsible for the occurrence of thrombocytopenia *in vivo* by activating platelets through interaction with platelet GPIb receptor or by mediating platelet adhesion to blood vessel walls. Detected snaclec peptides probably originate from snaclec subunits of the P-IIId SVMPs, which have been identified in the same fraction. The snaclec sequence AFCCPSGWSAYDQNCY is identical to the N-terminus of the light chain 1 (α -snaclec subunit) of the *Vaa* FX activator, VAFXA-II [26]. The other snaclec peptide was identified as part of the β -subunit of lebecin, a heterodimeric 30 kDa snaclec from *M. lebetina* [68], so the possibility that *Vbb* snaclecs form high molecular mass oligomers cannot be excluded [69].

4.2.8. Kunitz-type protease inhibitors (KPIs)

KPIs share a conserved fold of about 60 amino acid residues stabilized with three disulfide bridges. Besides inhibiting serine proteases,

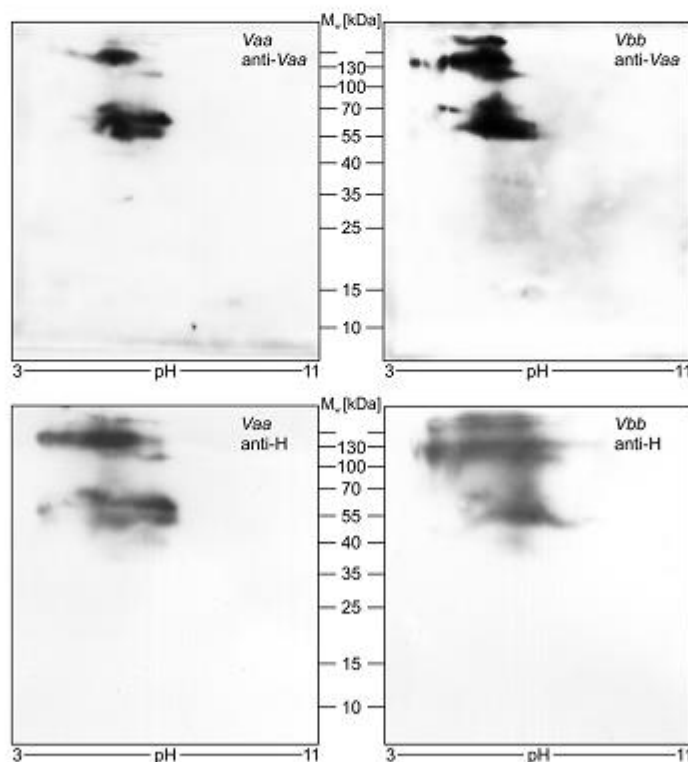


Fig. 9. Immunological comparison of the viper venoms. *Vaa* venom (left column) and the same amount (20 μ g) of *Vbb* venom (right column) were separated by electrophoresis in two dimensions, first in the pH gradient and then by molecular mass. From the gels proteins were electro-transferred on to PVDF membranes. For immuno-tagging the serum raised against the raw *Vaa* venom (anti-*Vaa*) (upper row), or the total haemorrhagic fraction of *Vaa* venom (anti-H) (lower row) was used. Visualization was achieved using HRP-conjugated anti-rabbit IgGs and the ECL detection system.

which could result in interference with the coagulation cascade, other KPIs, e.g. dendrotoxins and calciclaudine, block conductance of K^+ or Ca^{2+} ion channels. The presence of a KPI in Vbb venom was confirmed by N-terminal amino acid sequencing of peptides in the RP-HPLC fraction C18-10 (Fig. 6C, Table 2). One of the peptides in this fraction has the N-terminal amino acid sequence of the Vaa venom chymotrypsin inhibitor (VaaChi) [70]. VaaChi was first described by Ritonja et al. [71] and recently shown to form a complex with AtxA [72]. By itself, VaaChi is just a potent inhibitor of α -chymotrypsin ($K_i = 4.3$ nM) and is non-toxic to mouse, however, complexing to AtxA, appears to augment its toxicity [72]. The most recent demonstration of the snake venom toxicity-modulating role of a KPI was in *Micrurus tener* venom [73]. A KPI and an sPLA₂ molecule from this venom form a non-covalent complex that targets acid-sensing ion channels, producing pain. A similar function for Vbb venom KPI in generating pain is possible. Structurally, VaaChi belongs to the non-neurotoxic snake venom KPIs that have lost the ability to inhibit conductance of K^+ and Ca^{2+} channels [74]. Its amino acid sequence is 87% identical to that of *Macrovipera lebetina transmediterranea* venom KPI which is able to inhibit adhesion, migration and invasion of human glioblastoma U87 cells by binding to integrin receptors via the RGN motif [75]. The RGN motif is also present in VaaChi and, probably, also in Vbb KPI, suggesting a cancer inhibiting activity.

4.2.9. Natriuretic peptides (NPs)

NPs are polypeptide hormones about 40 amino acid residues long that elevate the level of natriuresis and diuresis in organisms [76]. In the case of Vbb venom we identified them in the RP-C18 fractions 6–9 (Fig. 6C, Table 2). The N-terminal sequences of peptides eluting in these fractions have been matched with part of the sequence of the Vaa NP precursor (KT148818) corresponding to the mature form of the NP. Snake venom NPs are typical inducers of systemic hypotension. Most probably, the NPs in the venom of Vbb are the main reason for the pronounced decrease of systemic blood pressure observed in patients envenomed by Vbb venom [14].

4.2.10. Disintegrins

Disintegrins are mono- or dimeric snake venom proteins without enzymatic activity. These cysteine-rich molecules of 4–14 kDa selectively bind integrin receptors on cells, obstructing their function [77]. In this way, they can affect, for example, the shaping, survival, proliferation, migration and invasion of cells [78]. A heterodimeric disintegrin, VB7, has been already described in Vbb venom [79] and its presence was confirmed in our proteomic survey. The N-termini of the two subunits were detected in the RP-C18 fraction 10 (Fig. 6B, Table 2). VB7 consists of a 64 amino acid residue subunit A linked to a 63 amino acid residue subunit B by interchain disulfide bonds. It binds strongly to the cell membrane

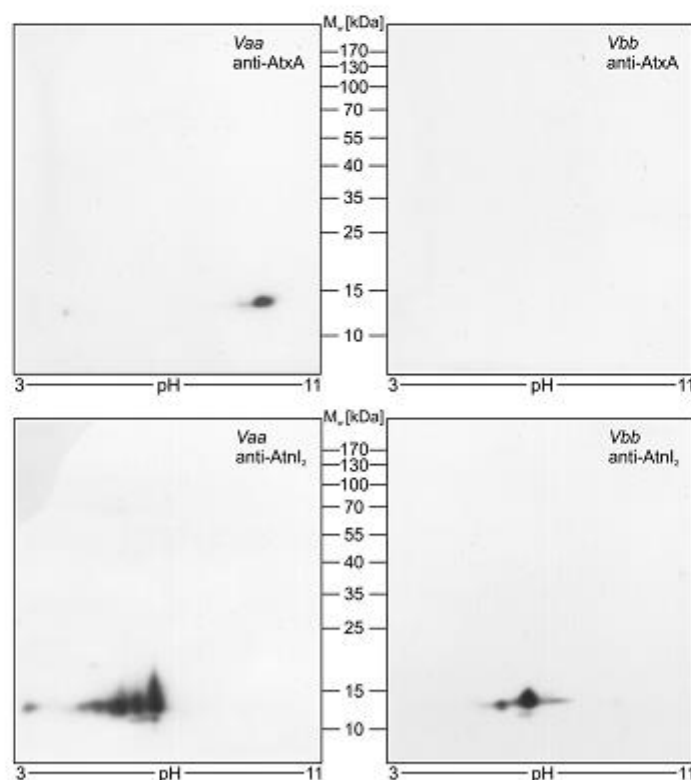


Fig. 10. Resemblance of sPLA₂s in both viper venoms. Vaa venom (left column) and the same amount (20 µg) of Vbb venom (right column) were separated by electrophoresis in two dimensions, according to pI and molecular mass. Proteins were Western blotted from the gels on to PVDF membranes. There the neurotoxic Atxs or the non-toxic Atns were tagged using specific anti-AtxA (upper row), or anti-AtnI₂ antibodies (lower row). Visualization was performed using HRP-conjugated anti-rabbit IgGs and the ECL detection system.

fibronectin-binding integrin $\alpha_5\beta_1$. In this way VB7 inhibits adhesion of the $\alpha_5\beta_1$ -expressing cells to fibronectin, a component of blood plasma and extracellular matrix [78]. Fibronectin plays a crucial role in wound healing and formation of a blood clot to stop bleeding and protect the underlying tissue, therefore, upon envenomation, VB7 supposedly hinders these processes.

The effects of viper venoms induced by their integrin-binding activity are, however, not associated only with disintegrins but also with their multidomain P-II and P-III SVMPS, which contain disintegrin, disintegrin-like and DC domains. These domains can be also released from their precursors by post-translational proteolytic processing [47]. In PLRP-S fraction 12, in the 33 kDa band, we detected for example a DC domain homologous to that of VbH4-B [25].

5. Conclusions

- Neurological disturbances are frequent in the case of poisoning by Vaa venom but only rare in the case of Vbb venom poisoning. The main neurotoxins of Vaa venom are ATxs, presynaptically neurotoxic sPLA₂s. These molecules were not detected in Vbb venom, either by specific antibodies or by sequencing. Although Vbb possesses genes encoding the neurotoxic sPLA₂s [56], these are obviously expressed only occasionally and to a very limited extent.
- Systemic haemorrhage and coagulopathy are usually much more severe in cases of Vaa poisoning than poisoning with Vbb venom. The haemorrhagic activity of Vaa venom is associated exclusively with the P-III SVMPS and, using proteomic and immunological studies, similar molecules are shown to be present in Vbb venom also. The less severe systemic haemorrhage in the case of Vbb venom poisoning is probably only the consequence of a lower concentration or activity of the respective haemorrhagic Vbb P-III SVMPS. Vaa venom degrades fibrinogen more intensively and less specifically than Vbb venom, although a similar effect on TT was observed in both cases. In contrast to Vbb venom, Vaa venom showed a pronounced anticoagulant effect founded on radical prolongation of the PT by this venom. This could be explained by the excessive consumption of coagulation factors [39] and by inhibition of the assembly/activity of the prothrombinase complex by ATxs through binding to FXa [80].
- Platelet aggregation and agglutination are completely inhibited by Vaa venom, but only partially by Vbb venom. Platelet aggregation/agglutination is inhibited by a range of snake venom proteins (SVMPS, sPLA₂s, LAOs, 5'-nucleotidases, disintegrins, snakelets and three-finger toxins), either by hydrolysis of ADP, by degradation of platelet receptors involved in these processes, or by antagonising collagen- or von Willebrand factor receptor binding [41]. Our study exposed snakelets as one of the most possible factors of such difference between both venoms. Classic snakelets, which are abundantly present in Vaa venom [39], have namely not been found in Vbb venom at all.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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3.2 Znanstvena publikacija 2: Kardiotoksični učinki frakcij modrasovega strupa v izoliranem perfuziranem srcu podgane

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V dosedanjih kliničnih poročanjih so bili opisani primeri pacientov po ugrizu kač iz poddružine Viperinae (*Vaa*, *Vbb* in *D. russelii*), ki so imeli izrazite vplive na delovanje srčne mišice, kot na primer: motnje srčnega ritma, arterijske hiper- in hipotenzije, srčno kap in ne-bakterijski endokarditis. V poskusih, kjer so modrasov strup vbrizgali v živali, so opazili hiter padec aortnega tlaka z aritmijami, a so ti poskusi potekali pod tehnično nenadzorovanimi pogoji. Da bi zagotovili natančno določene pogoje dela, smo za svoj študij kardiotoksičnosti modrasovega strupa izbrali izolirano in perfuzirano podganje srce, *ex vivo* model po Langendorffu.

S pomočjo gelske kromatografije smo modrasov strup ločili na štiri večje frakcije (A, B, C in E) ter jih analizirali s pomočjo NaDS-PAGE. Frakcija A je vsebovala proteine z najvišjo navidezno molekulsko maso (60–100 kDa), frakcija B proteine z maso 30–40 kDa, frakcija C pa tiste z maso 14–15 kDa. Na osnovi predhodne študije [12] smo vedeli, da frakcija E vsebuje predvsem tripeptide pEKW, ki so inhibitorji MP, zato je nismo testirali na podganjem srcu.

Izmed treh testiranih frakcij je frakcija C najbolj izrazito znižala srčni utrip. Pri višjih koncentracijah frakcije C (22,5 in 37,5 µg/ml) je v dveh min po vbrizgu nastopil srčni zastoj zaradi ireverzibilne mišične kontrakcije. Frakcija C je tudi zvišala levi diastolični ventrikularni tlak (dLVP) in hkrati znižala levi sistolični ventrikularni tlak (sLVP). Frakcija B je le ob najvišji aplicirani koncentraciji 37,5 µg/ml sprva povzročila upočasnitev srčnega utripa, kasneje pa ventrikularno tahikardijo, čemur je sledila ventrikularna fibrilacija in asistola. Frakcija A ni imela vpliva na utrip izoliranih src, na koronarni tok ter na tlak v levem ventriklu.

Moj prispevek v znanstveni publikaciji je bilo ločevanje modrasovega strupa, karakterizacija posameznih frakcij in njihova priprava za poskus na podganjem srcu ter pomoč pri pisanju eksperimentalnih metod.

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Cardiotoxic effects of the *Vipera ammodytes ammodytes* venom fractions in the isolated perfused rat heart



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ABSTRACT

The nose-horned viper (*Vipera ammodytes ammodytes*) is the most venomous European snake. Its venom is known as haematotoxic, myotoxic and neurotoxic but it exerts also cardiotoxic effects. To further explore the cardiotoxicity of the venom we separated it into four fractions by gel filtration chromatography. Three fractions that contain polypeptides (A, B, and C) were tested for their effects on isolated rat heart. Heart rate (HR), incidence of arrhythmias (atrioventricular (AV) blocks, ventricular tachycardia, ventricular fibrillation, and asystolia), coronary flow (CF), systolic, developed and diastolic left ventricular pressure (LVP) were measured before, during, and after the application of venom fractions in three different concentrations. Fraction A, containing proteins of 60–100 kDa, displayed no effect on the rat heart. Fractions B and C disturbed heart functioning in similar way, but with different potency that was higher by the latter. This was manifested by significant decrease of HR and CF, the increase of diastolic, and the decrease of systolic and developed LVPs. All hearts treated with fraction C in the final CF concentrations 22.5 and 37.5 µg/mL suffered rapid and irreversible asystolia without AV blockade. They underwent also ventricular fibrillation and ventricular tachycardia. Fraction B affected hearts only at the highest dose inducing asystolia in all hearts, ventricular fibrillation in 80% and ventricular tachycardia in 70% of the hearts. Venom fraction C induced 71% of all recorded heart rhythm disturbances, significantly more than fraction B, which induced 29%. Most abundant proteins in fraction C were secreted phospholipases A₂ among which the venom component acting on the heart is most probably to be looked for.

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1. Introduction

The nose-horned viper, *Vipera ammodytes ammodytes* (V. a. *ammodytes*) is the most venomous viper in Europe (Lukšić et al., 2006; Chippaux, 2012; Karabuya et al., 2016). Poisoning by V. a. *ammodytes* venom can vary from mild to severe, with obvious systemic toxicity, and be even fatal. The more severe cases of poisoning have been recorded especially when the venom was

injected directly into the bloodstream of the victim or in the vicinity of his heart (Reid, 1976; Persson and Irestedt, 1981; Lukšić et al., 2010). V. a. *ammodytes* venom is a complex mixture of enzymatic and non-enzymatic proteins and peptides with diverse pathophysiological activities (Georgieva et al., 2008; Križaj, 2011; Sajevec et al., 2013) leading to several clinical appearances, most characteristic, neurotoxic and haematotoxic (Lukšić et al., 2006; Karabuya et al., 2016).

Neurological signs have been described as the most common general complication in children and in adults in the case of the V. a. *ammodytes* envenomation (Lukšić et al., 2006; Chippaux, 2012; Karabuya et al., 2016). Descriptions of neurological effects have been frequently found also in case reports of envenomation by Viperinae snakes closely related to the V. a. *ammodytes* (Ferquel

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et al., 2007; De Haro et al., 2009; Malina et al., 2013; Lonati et al., 2014; Jollivet et al., 2015; Karabuvu et al., 2016). Proteins that affect the nervous system of the victim have been most extensively characterized in the *V. a. ammodytes* venom by biochemical, molecular biology, immunological and other methods, and are very well known (Križaj, 2011; Šribar et al., 2014).

Disturbances of the haemostatic system with systemic effects like haemorrhagic syndrome with massive intravascular haemolysis, pulmonary bleeding, severe coagulopathy, and other haemodynamic alternations have been frequently described at envenoming by the *V. a. ammodytes* and other *Viperinae* venoms (Ozay et al., 2005; White, 2005; Lukšić et al., 2006, 2010; De Haro et al., 2009; Karabuvu et al., 2016). Components of the *Viperinae* venoms that affect normal functioning of the haemostatic system are already well known, biochemically and structurally, and their actions are mainly mechanistically explained (Markland, 1998; Gutiérrez et al., 2005; Sajević et al., 2011, 2013).

Several case reports about patients suffering from a *Viperinae* venomous bite are describing vessel and myocardial dysfunctions, for example, different rhythm abnormalities, arterial hypertension and hypotension also, ST elevation myocardial infarction, non-bacterial thrombotic endocarditis, and states of threatening shock (Moore, 1988; Singh et al., 1998; Lukšić et al., 2006, 2010; Malina et al., 2008; Silva et al., 2012). Experimental animals poisoned with *Viperinae* venom, in which cardiovascular repercussions have been registered, usually manifested a rapid fall of aortic pressure with rhythm abnormalities (Valora et al., 1970; Sket et al., 1973; Kinjo et al., 1990; Alloatti et al., 1991; Fatehi-Hassanabad and Fatehi, 2004). Available studies are however not very reliable as they were usually conducted in technically uncontrolled systems and using non-standardized venoms.

We described previously dose-dependent cardiotoxic effects of the whole standardized crude *V. a. ammodytes* venom on isolated rat heart asserting the depression of contractility and electrophysiological instability (Lukšić et al., 2008). To further explore these effects we separated the crude *V. a. ammodytes* venom into several fractions, and tested individual fractions for their effects on different parameters of cardiac function in the model of isolated perfused rat heart. *Viperinae* venom components that affect the heart function are unknown. This study is therefore also a starting point towards their description.

2. Materials and methods

2.1. Materials

Crude *V. a. ammodytes* venom was procured from the Institute of Immunology Inc., Zagreb, Croatia. Venom was collected by "milking" snakes from regions of Krapina (70%) and Samobor (30%). The crude venom was dried at the ambient temperature, lyophilized and stored in dry, dark bottles at -20°C . All other chemicals were of analytical and sequencing grade.

2.2. Methods

2.2.1. Gel filtration chromatography of the crude *V. a. ammodytes* venom

Crude venom (1 g) was dissolved in 15 mL of 20 mM Tris buffer, pH 7.0, containing 2 mM CaCl_2 and 300 mM NaCl, and separated at 4°C on a Sephacryl S-200 superfine column (100×4 cm) equilibrated in the same buffer, at a flow rate of 33.6 mL/h. Eight mL fractions were collected and the absorbance was measured at 280 nm (A_{280}). Fractions from the same peak were pooled and concentrated.

2.2.2. SDS-polyacrylamide gel electrophoresis

Protein compositions of concentrated gel filtration fractions were analysed by SDS-PAGE on 12.5% (w/v) polyacrylamide gel under non-reducing conditions according to Laemmli (Laemmli, 1970). Molecular mass standards were from Fermentas (Vilnius, Lithuania). Gel-separated proteins were stained with colloidal silver.

2.2.3. Concentrations of proteins in the venom fractions used in testing

Lukšić et al. reported cardiotoxic effects in isolated rat heart upon usage of 3, 9, and 15 mg/mL stock solution of the raw *V. a. ammodytes* venom, which resulted in final coronary flow (CF) venom concentrations 30, 90, and 150 $\mu\text{g/mL}$, respectively (Lukšić et al., 2008). With an estimation that each venom gel filtration fraction (A, B, and C) contains 25% of the whole venom protein content we used in our assays 0.75, 2.25, and 3.75 mg/mL stock solutions of each fraction in the modified Krebs-Henseleit solution. Stock solutions of fractions were infused into the rat hearts for 10 min at the rate of 1% of CF to get the final CF venom protein concentrations of 7.5, 22.5, and 37.5 $\mu\text{g/mL}$.

2.2.4. Preparation of the rat heart and measurement of cardiac functions

Ex vivo cardiac measurements were done on Langendorf model of isolated perfused rat heart according to the same preparation conditions described in research of Lukšić et al. (Lukšić et al., 2008). Sinus rhythm was considered to be a physiologically normal rhythm in the baseline conditions, and heart rate (HR) in this research was measured according to noted rhythm. AV conduction time was determined as the interval between the superior right atrial beat and the right ventricular conus beat. In cases of prolonged AV conduction time, AV blocks were noted as following: first-degree AV block (delayed impulse conduction from atria to ventricles), second-degree AV block (condition when one or more, but not all of the atrial impulses fail to conduct to the ventricles), third-degree (complete) AV block (impulses generated in the atrium do not propagate to the ventricles). Ventricular tachycardia (VT) was defined as four or more consecutive uniform or multiform ventricular waveforms, and a faster ventricular than atrial rate. Ventricular fibrillation (VF) was registered by the presence of irregular activity in the ventricular electrocardiogram, and by the absence of pressure generation by the left ventricle. The absence of electrical and mechanical activity of the heart was considered as asystolia (asystolic cardiac arrest).

2.2.5. Experimental protocol

The control group of isolated perfused rat hearts ($N = 10$) was not treated with any substance, and was monitored for 70 min. Groups of treated hearts (total $N = 90$) were first monitored for 30 min before venom fraction application, then the period of venom fraction delivery started (the 30th min after the beginning of the experiment) and lasted for the next 10 min. During this period, each venom fraction (A, B, and C) was administered in three different doses (refer to section 2.2.3.). All parameters of the cardiac function were registered at the beginning of the venom fraction delivery (0 min), then in the 3rd min of the venom fraction delivery, and finally in the 13th min past the start of the venom fraction delivery, i.e. in the 3rd min after the cease of the venom fraction delivery. In the end, observation period lasted for 30 min. Complete experiment therefore lasted 70 min. Procedures and all handling with animals were in strict accordance with the EU Directive 86/609/EEC, and Convention 123, the Directive 2010/63/EU. Ethics Committees of the Mostar University School of Medicine and Split

University School of Medicine approved all experiments on animals.

2.2.6. Statistical analysis

Categorical variables are presented as counts (N) and percentages, and were calculated using χ^2 -test. Quantitative data are expressed as mean $\pm$ standard deviation (mean $\pm$ SD), and were assessed with analysis of variance (ANOVA) and Kruskal-Wallis test, followed by Bonferroni or Tukey's post-hoc multiple comparisons. Data were processed using SPSS Statistics (version 14.0, SPSS INC., Chicago, Illinois, USA). P value < 0.01 was considered as statistically significant.

3. Results

3.1. Preparation and characterisation of the venom fractions

Using gel filtration chromatography the crude *V. a. ammodytes* venom was separated in four fractions: A, B, C, and E (Fig. 1). Their SDS-PAGE analysis under non-reducing conditions is shown in the inset of Fig. 1. Fraction A was abundant in venom proteins with apparent molecular masses of 60–100 kDa. The majority of proteins in fraction B displayed masses in the range 30–40 kDa, while fraction C contained 14–15 kDa proteins. In our previous study we showed that fraction E contains mainly a tripeptide pEKW, an inhibitor of snake venom metalloproteinases (SVMPs) (Sajevic et al., 2013), therefore, this fraction has not been considered in our study.

3.2. Effects of the venom fractions on the rat heart ex vivo

3.2.1. Electrophysiological effects

Sinus rhythm was predominant in all hearts in the beginning of an experiment (Table 1). Fraction C induced the most pronounced effect on frequency of the isolated perfused rat heart beat (Table 1 and Fig. 1SA). Hearts exposed to the final CF concentration 7.5 $\mu\text{g/mL}$ of fraction C showed significant slowing of the HR as measured in the 3rd and 13th min from the start of the fraction delivery in comparison with the baseline heart frequency $P < 0.01$.

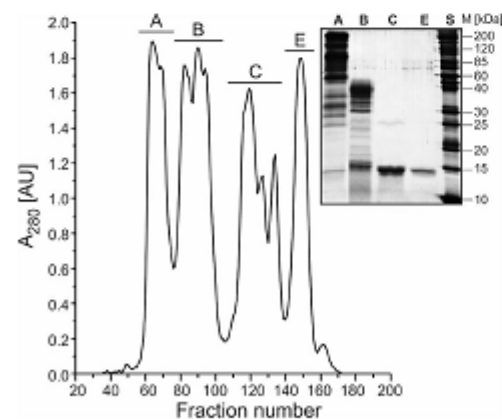


Fig. 1. Gel filtration chromatography of the *V. a. ammodytes* venom. One g of the raw venom was separated on the Sephacryl S-200 Superfine column. Fractions (A, B, C, and E) were collected as depicted. Inset: SDS-PAGE analysis of the venom fractions under non-reducing conditions. The gel is silver stained. M: molecular mass, S: protein standards, A–E: venom fractions.

Particularly interesting is a significant decrease in the 13th vs. 3rd min ($P < 0.01$; Table 1 and Fig. 1SA), this is 3 min after the end of the venom fraction delivery. During measurements, sinus rate was the prime rhythm, and no change of the rhythm type was observed. HR that was lower in the period of the venom fraction exposure remained the same during the observation period, and did not recover until the end of experiment (Fig. 1SA). After administering fraction C in the final CF doses of 22.5 and 37.5 $\mu\text{g/mL}$, cardiac arrest was registered in all hearts in ~ 2 min (Fig. 1SA and Fig. 2), which was confirmed with statistical significance ($P < 0.01$). In all cases ($N = 20$) of cardiac arrest, ventricular tachycardia, and ventricular fibrillation preceded asystolia (Fig. 2). Fraction B in the final CF concentration of 7.5 $\mu\text{g/mL}$ caused clinically insignificant variations of HR (Table 1 and Fig. 1SB). Increase of the CF concentration of this fraction to 22.5 $\mu\text{g/mL}$ induced significant but reversible decrease of HR and no changes of the rhythm type ($P < 0.01$; Table 1 and Fig. 1SB). On the contrary, in concentration of 37.5 $\mu\text{g/mL}$ this fraction caused significant slowing of the sinus HR in the 3rd min from the beginning of the sample delivery, and then, ~ 12 min past the application of the sample, complete arrest of electrophysiological activity of the heart ($P < 0.01$; Table 1 and Fig. 1SB). Hearts did not recover until the end of the measurement so the effect was irreversible (Fig. 1SB). In these cases, before cardiac arrest, sinus rhythm changed into ventricular tachycardia (7 out of 10), followed by ventricular fibrillation (8 out of 10) that very quickly led to asystolia in all exposed hearts ($N = 10$) (Fig. 2). Sinus rhythm was also the main electrical activity in all hearts in the period during and after exposure to fraction A. No statistically or clinically significant change in HR was observed at any concentration of fraction A tested (Table 1). As well, no arrhythmias were recorded with fraction A (Fig. 2). These were significantly more frequent at 37.5 $\mu\text{g/mL}$ of fraction B and C ($P < 0.01$) than at 22.5 $\mu\text{g/mL}$ (Fig. 2). Of altogether 85 heart rhythm disturbances documented, fraction C was responsible for 71% and fraction B for 29% ($P < 0.01$). Any case of AV block was detected.

3.2.2. Coronary flow

At the beginning of the observation the CF was about the same in all hearts (Table 1). Most significantly the CF was affected by the venom fraction C, at all three doses (Table 1 and Fig. 2SA). Hearts exposed to the final CF concentrations 22.5 and 37.5 $\mu\text{g/mL}$ of fraction C displayed irreversible muscle contracture from ~ 2 nd min of the exposure on (Fig. 2SA). This led to precluded coronary circulation demonstrated as significant decrease of the CF values ($P < 0.01$; Table 1). After application of 7.5 $\mu\text{g/mL}$ of fraction C, CF also significantly decreased ($P < 0.01$) and remained low until the end of the measurement (Fig. 2SA). Exposure of hearts to the final CF dose of 7.5 $\mu\text{g/mL}$ of fraction B did not result in important changes in the CF (Fig. 2SB). However, 22.5 $\mu\text{g/mL}$ of fraction B significantly decreased CF in the 13th min ($P < 0.01$), this is 3 min after the venom fraction delivery has been interrupted. Interestingly, further on the coronary circulation started to recover slightly but steadily (Fig. 2SB). At the highest final CF concentration (37.5 $\mu\text{g/mL}$) fraction B significantly decreased the CF due to the cardiac arrest that occurred ~ 12 min past the start of hearts exposure. Statistically significant ($P < 0.01$), thereafter hearts stayed permanently contracted (Fig. 2SB). Fraction A did not induce any significant change of the CF (Table 1).

3.2.3. Left ventricular pressures

Fraction A was without an effect on the devLVP, the dLVP and the sLVP at all concentrations tested, while fraction C induced the most significant variation of these parameters. At the final CF dose of 7.5 $\mu\text{g/mL}$, fraction C induced a slight decrease in the devLVP; however, at doses 22.5 and 37.5 $\mu\text{g/mL}$, an abrupt drop of this

Table 1

Cardiac parameters measured in the isolated perfused rat hearts exposed to the *V. a. ammodytes* venom gel filtration chromatography fractions (Fig. 1). All values are expressed as means $\pm$ SD of ten independent measurements, and calculated using ANOVA.

Fraction [$\mu\text{g/mL}$]	Time [min]	Cardiac parameters (mean $\pm$ SD)				
		HR ^a [beats/min]	CF ^b [mL/min]	sLVP ^c [mmHg]	devLVP ^d [mmHg]	dLVP ^e [mmHg]
Control		289.6 $\pm$ 1.6	11.1 $\pm$ 0.1	116.0 $\pm$ 2.3	116.0 $\pm$ 2.3	0
A [7.5]	0	289.8 $\pm$ 1.5	11.1 $\pm$ 0.1	116.5 $\pm$ 2.5	116.5 $\pm$ 2.5	0
	3	288.3 $\pm$ 1.5	11.1 $\pm$ 0.1	115.8 $\pm$ 1.7	115.8 $\pm$ 1.7	0
	13	286.3 $\pm$ 1.5	11.1 $\pm$ 0.1	116.0 $\pm$ 2.2	116.0 $\pm$ 2.2	0
A [22.5]	0	295.8 $\pm$ 2.5	11.2 $\pm$ 0.2	116.8 $\pm$ 2.8	116.8 $\pm$ 2.8	0
	3	291.8 $\pm$ 2.9	11.2 $\pm$ 0.2	115.3 $\pm$ 1.3	115.3 $\pm$ 1.3	0
	13	289.5 $\pm$ 2.6	11.2 $\pm$ 0.1	114.8 $\pm$ 1.7	114.8 $\pm$ 1.7	0
A [37.5]	0	292.8 $\pm$ 1.7	11.1 $\pm$ 0.2	117.8 $\pm$ 1.7	117.8 $\pm$ 1.7	0
	3	291.5 $\pm$ 1.3	11.1 $\pm$ 0.3	115.3 $\pm$ 1.3	115.3 $\pm$ 1.3	0
	13	288.0 $\pm$ 2.2	11.1 $\pm$ 0.2	114.5 $\pm$ 1.3	114.5 $\pm$ 1.3	0
Control B [7.5]		293.1 $\pm$ 2.5	11.1 $\pm$ 0.1	116.0 $\pm$ 1.8	116.0 $\pm$ 1.8	0
	0	293.3 $\pm$ 3.0	11.1 $\pm$ 0.2	116.5 $\pm$ 1.3	116.5 $\pm$ 1.3	0
	3	290.8 $\pm$ 2.1	11.2 $\pm$ 0.2	116.3 $\pm$ 1.3	116.3 $\pm$ 1.3	0
B [22.5]	0	287.5 $\pm$ 2.1	11.0 $\pm$ 0.1	114.5 $\pm$ 1.3	114.5 $\pm$ 1.3	0
	0	293.8 $\pm$ 3.9	11.0 $\pm$ 0.3	117.0 $\pm$ 2.2	117.0 $\pm$ 2.2	0
	3	275.8 $\pm$ 4.6 ^f	9.4 $\pm$ 1.1	115.3 $\pm$ 2.1	114.0 $\pm$ 2.2	1.3 $\pm$ 0.5
B [37.5]	0	250.3 $\pm$ 4.3 ^f	8.1 $\pm$ 0.2 ^f	97.0 $\pm$ 2.2 ^f	95.5 $\pm$ 2.1 ^f	1.5 $\pm$ 0.6
	3	293.3 $\pm$ 3.9	11.2 $\pm$ 0.2	116.8 $\pm$ 1.0	116.8 $\pm$ 1.0	0
	13	235.0 $\pm$ 6.7 ^f	9.1 $\pm$ 1.0 ^f	132.8 $\pm$ 2.2 ^f	92.5 $\pm$ 1.3 ^f	40.3 $\pm$ 1.0 ^f
Control C [7.5]		1.0 $\pm$ 0.0 ^g	4.6 $\pm$ 0.3 ^g	129.5 $\pm$ 1.7 ^g	92.8 $\pm$ 1.0 ^g	36.8 $\pm$ 1.0 ^g
	0	292.2 $\pm$ 2.4	11.1 $\pm$ 0.1	115.9 $\pm$ 1.2	115.9 $\pm$ 1.2	0
	0	292.0 $\pm$ 2.2	11.1 $\pm$ 0.3	115.8 $\pm$ 1.0	115.8 $\pm$ 1.0	0
C [22.5]	3	255.0 $\pm$ 3.6 ^f	9.9 $\pm$ 0.7	96.8 $\pm$ 1.0 ^f	94.0 $\pm$ 0.8 ^f	2.8 $\pm$ 0.5
	13	221.5 $\pm$ 4.2 ^f	8.6 $\pm$ 0.7 ^f	97.0 $\pm$ 1.6 ^f	94.3 $\pm$ 1.3 ^f	2.8 $\pm$ 0.5
C [37.5]	0	293.3 $\pm$ 3.2	11.1 $\pm$ 0.2	115.5 $\pm$ 1.3	115.5 $\pm$ 1.3	0
	3	1.0 $\pm$ 0.0 ^g	4.5 $\pm$ 0.7 ^g	47.8 $\pm$ 1.5 ^g	1.3 $\pm$ 0.5 ^g	46.5 $\pm$ 1.3 ^g
	13	1.0 $\pm$ 0.0 ^g	3.8 $\pm$ 0.2 ^g	47.0 $\pm$ 1.8 ^g	1.5 $\pm$ 0.6 ^g	45.5 $\pm$ 1.3 ^g
C [37.5]	0	292.5 $\pm$ 4.2	11.1 $\pm$ 0.1	117.5 $\pm$ 1.3	117.5 $\pm$ 1.3	0
	3	1.0 $\pm$ 0.0 ^g	4.5 $\pm$ 0.3 ^g	52.0 $\pm$ 1.6 ^g	1.3 $\pm$ 0.5 ^g	50.8 $\pm$ 1.3 ^g
	13	1.0 $\pm$ 0.0 ^g	3.7 $\pm$ 0.5 ^g	52.3 $\pm$ 1.0 ^g	1.0 $\pm$ 0.0 ^g	51.3 $\pm$ 1.0 ^g

^fP < 0.01 vs. control cardiac parameters.

^a HR: heart rate.

^b CF: coronary flow.

^c sLVP: systolic left ventricular pressure.

^d devLVP: developed left ventricular pressure.

^e dLVP: diastolic left ventricular pressure.

parameter to zero value resulted in ~ 2 min following the application (Fig. 3SA). In the case of fraction B, at the highest final CF concentration of 37.5 $\mu\text{g/mL}$, the decrease of the devLVP was comparable to that observed at the lowest final CF concentration (7.5 $\mu\text{g/mL}$) of the venom fraction C (Table 1 and Fig. 3S). Interestingly, at an intermediate dose of fraction B (22.5 $\mu\text{g/mL}$), the devLVP first decreased and after ~ 10 min started to rise again towards the initial level (Fig. 3SB). The change in the dLVP was opposite to the change of the devLVP in the presence of increasing final CF concentrations of fractions B and C (Table 1 and Fig. 4S). The dLVP of the isolated perfused rat hearts was increasing in the presence of the increasing concentrations of fractions B and C. Again, the effect was more pronounced in the case of fraction C. Significant decrease of the sLVP and the devLVP in the 3rd min of heart treatment and the 3rd min past the treatment was linked with rising of dLVP even with the lowest dose (7.5 $\mu\text{g/mL}$) of fraction C ($P < 0.01$ Figs. 3SA and 4SA). In experiments with the final CF concentrations of 22.5 and 37.5 $\mu\text{g/mL}$ of fraction C the devLVP was almost immeasurable due to the extreme rise of the dLVP (Figs. 3SA and 4SA), which was

observed as significant change vs. baseline conditions ($P < 0.01$; Table 1). This is in accordance with irreversible heart contracture that occurred during its exposure to fraction C in these doses. Fraction B induced significant and irreversible changes in sLVP and devLVP, and a high increase of the devLVP only at the final CF concentration of 37.5 $\mu\text{g/mL}$ ($P < 0.01$), where all exposed hearts remained in an irreversible contracture state, and finally experienced asystolic arrest in ~ 12 th min after the point of application (Figs. 3SB and 4SB). Upon exposure of rat hearts to 22.5 $\mu\text{g/mL}$ slight rising of the dLVP was noticed in the 3rd min of the exposure that still persisted in the 3rd min after the exposure. This was accompanied by a significant lowering of the sLVP ($P < 0.01$) and the devLVP ($P < 0.01$; Table 1). All mentioned changes were reversible (Figs. 3SB and 4SB). The final CF fraction dose of 7.5 $\mu\text{g/mL}$ did not induce changes in LVPs (Table 1).

4. Discussion

Regarding significant medical importance of envenomation by

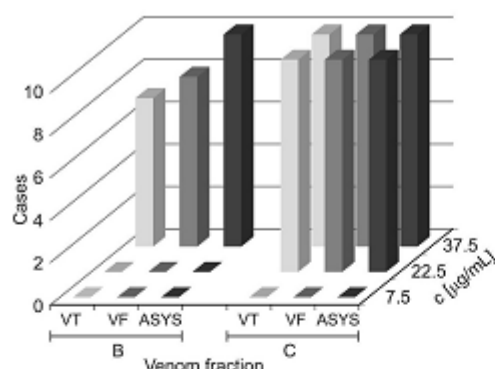


Fig. 2. Dose-dependent incidence of rhythm disturbances of the isolated perfused rat hearts by the *V. a. ammodytes* venom fractions. Isolated rat hearts were infused with the venom gel filtration chromatography fractions A, B, and C in the final coronary flow concentrations; 7.5, 22.5, and 37.5 µg/mL. With each fraction at each dose the experiment was performed on ten hearts. Fraction A did not affect rat hearts at all. Incidence of heart arrhythmias: ventricular tachycardia (VT), ventricular fibrillation (VF), and asystolia (ASYS), in the case of fraction B and C is displayed.

the *V. a. ammodytes* venom in particular areas of Europe, for example in Mediterranean countries (Lukšić et al., 2006; Karabuvu et al., 2016), we aimed in this work to further dissect cardiotoxic effects of the raw venom that were reported before (Lukšić et al., 2008). To this end we separated the venom using gel filtration chromatography into four fractions (Fig. 1). The influence on an array of cardiac parameters of the fractions that contain proteins (fractions A, B, and C) has been evaluated and related to their protein compositions (Georgieva et al., 2008; Sajevec et al., 2013).

Concentrating on the cardiovascular effects of *Viperidae* venoms, the previously conducted studies were focused on basic electrocardiographic changes with no attention on malignant rhythm changes and with only a few measured parameters that are clinically classified as cardiotoxic (Kinjo et al., 1990; Alloatti et al., 1991). As well, in these studies non-standardized venoms were used in uncontrolled systems together with potentially cardio-protective agents aiming to search for pharmacological tools of venom neutralization. Experimental model that we have employed in our study was a controlled system of the isolated perfused rat heart (Lukšić et al., 2008). The model has some limitations, which one has to be aware of at interpretation of the results. The isolated heart cannot respond to envenomation in the same way as the complete organism would respond. For example, in cases of haemodynamic instability the organism activates compensatory mechanisms, like renal arteries and peripheral circulation resistance to support cardiac insufficiency, which is obviously not possible in the case of the isolated heart.

The venom fraction A was not cardiotoxic in our model. The constituents of this fraction are proteins of 60–100 kDa, mainly class P-III SVMPs, haemorrhagic and non-haemorrhagic, and L-amino acid oxidases (Leonardi et al., 2007, 2008; Georgieva et al., 2008; Sajevec et al., 2013; Leonardi et al., 2015).

Fraction B, composed of 30–40 kDa venom proteins, fully expressed its cardiotoxicity only at the highest final CF dose tested (37.5 µg/mL). Infused by such concentration, each heart experienced arrhythmias and suffered asystolia. Arrhythmogenic effect may be caused by (myo)toxic components of the fraction B that induced hypercontracture state of myocardium, and arrest together with aberrant conduction pathways formed in the

damaged heart. Intermediate dose of fraction B, 22.5 µg/mL, influenced heart function reversibly. This could be explained by predominately haematotoxic nature of fraction B (Sajevec et al., 2013). Reversible effect on heart action was reported also by Lukšić et al. when they exposed rat hearts to the lowest final CF dose of 30 µg/mL of the raw *V. a. ammodytes* venom (Lukšić et al., 2008). Observation of two types of heart effects, reversible and irreversible, can be interpreted by the presence of components in the *V. a. ammodytes* venom that cause reversible cardiotoxicity and the components that induce irreversible damage to the heart. The latter is in accordance with the report of De Haro et al. who observed at autopsy a severe myocardial damage following envenomation by the venom of the closely related *Vipera aspis* snake (De Haro et al., 2009). Alternatively, reversibility of the cardiotoxic effect may depend on the concentration of cardiotoxic component. Proteins of various protein families are present in the venom fraction B: snake venom serine proteases (SVSPs), class P-I SVMPs, cysteine-rich secretory proteins (CRISPs), snake C-type lectins (snaclecs), and disintegrins (Georgieva et al., 2008; Sajevec et al., 2013). Myotoxic activity of fraction B, the most likely cause of its cardiotoxicity, may be the result of CRISPs that inhibit depolarization-induced contraction of arterial smooth muscle by interacting with voltage-gated Ca^{2+} and K^{+} channels, and also by blocking cyclic nucleotide-gated channels (Yamazaki et al., 2003; Yamazaki and Morita, 2004; Ramazanov et al., 2009). Myotoxic activity of this fraction likely stems also from ammodytin L, the main myotoxin of the venom. It has a structure of secreted phospholipase A₂ (sPLA₂) (Križaj et al., 1991) with ~14 kDa, but as it is prone to form a dimer it is surely present to some extent also in fraction B. SVSPs, SVMPs, snaclecs, and disintegrins in fraction B, as far as we know, influence in the first place haemostatic system (Sajevec et al., 2013), but cardiotoxic activity of some of these molecules cannot be excluded.

The large majority of cardiotoxicity of the *V. a. ammodytes* venom was associated with its gel filtration chromatography fraction C. Cardiotoxicity was revealed as significant decrease of HR and CF, together with the increase of the dLVP joined with the decrease of sLVP and devLVP. Rat hearts exposed to the venom fraction C in final CF concentrations of 22.5 and 37.5 µg/mL suffered irreversible asystolic cardiac arrest already after ~2 min. As well, fraction C induced significantly more rhythm disturbances than other venom fractions. Cardiotoxic effects induced by the venom fraction C were very similar to those reported for the raw venom in the same experimental system (Lukšić et al., 2008), suggesting that the main cardiotoxic factors of the venom are in this fraction. The most prominent effect reported was a depression of the heart contractile function, linked to significant dose-dependent increase of biochemical markers due to the heart damage. The same study also reported about electrophysiological abnormalities manifested as short period of increase of HR followed by its dose-dependent slow down (Lukšić et al., 2008). Such effects were also observed with crude *Vipera lebetina* and *Bitis nasicornis* venoms (Alloatti et al., 1986, 1991; Fatehi-Hassanabad and Fatehi, 2004). Since positive chronotropic effects were prevented by propranolol and ranitidine, β -adrenergic and histamine receptor blockers, it was suggested that the activities were mediated by catecholamines and histamine released from the heart (Alloatti et al., 1986, 1991; Fatehi-Hassanabad and Fatehi, 2004). Rhythm abnormalities, like AV blocks, and ventricular malignant arrhythmias that preceded asystolia were also registered in Lukšić et al. study where prolongation of AV conduction time during toxic exposure was permanent and dose-dependent (Lukšić et al., 2008). We did not detect prolongation of AV conduction time, and ventricular tachycardia and fibrillation along with asystolia occurred more often than reported by Lukšić et al. for the whole venom (Lukšić et al., 2008). Observed

differences in effect on rat heart between the whole venom and its isolated fraction C are likely the result of combination of effects of more components in the case of crude venom, some of which were absent in fraction C. Increase of dLVP and decrease of sLVP was similar if the rat heart was perfused with the crude venom (Lukšić et al., 2008) or its fraction C.

Profound decrease of CF registered during exposure to our fraction C and also to the raw venom (Lukšić et al., 2008) was not a result of the direct damage of the venom to coronary vessels. It is probably the consequence of the myocardial contracture and precluded coronary circulation since heart contracture happened before blood flow through coronary arteries stopped. The proposed mechanism for explaining the effect of the venom fraction C on myocardium contractility is impairment of intracellular Ca^{2+} channels that results in uncontrolled input of Ca^{2+} leading to elevation of cytosol Ca^{2+} in the myocytes, primarily as a result of cell membrane damage, which in the end induces contracture of the heart muscle and increases heart energy expenditure that worsens ventricular contracture (Lukšić et al., 2008; Montecucco et al., 2008). Lukšić et al. reported that oxygen myocardial consumption remained relatively high in comparison to the greater contractility depression indicating expenditure of oxygen and energy in hearts envenomed with the raw venom (Lukšić et al., 2008).

Fraction C harbours proteins of ~14 kDa. These are mostly sPLA₂s, neurotoxic ammodytoxins (Križaj, 2011), non-toxic ammodytoxins (Križaj et al., 1992), and enzymatically inactive myotoxic ammodytin I (Križaj et al., 1991). As classical cardiotoxins, toxins of ~6 kDa with a three-finger fold (Kini and Doley, 2010), are not present in the venom of the *V. a. ammodytes* (Georgieva et al., 2008; Sajevec et al., 2013), it is most likely that cardiotoxicity of the venom is induced by one or more sPLA₂ molecules in the venom. Cytolytic action of a cardio-active venom component is inferred, as the level of biochemical markers in the heart effluent increased dependently on the venom dose (Lukšić et al., 2008). Based on indirect experimental evidence, a myotoxic sPLA₂ analogue ammodytin I is most likely one of the venom protein with cardiotoxic activity. Interactions of some snake venom sPLA₂ molecules with different isolated cell types are well known (Kini, 2003; Doumanov et al., 2014), and the direct action on heart has been also indicated by other authors: the sPLA₂ from *Naja nigricollis* venom induced hypotension and bradycardia with a block in electrical conduction in the myocardium (Barrington et al., 1986), while the one from *Bothrops marajoensis* venom shortened, independently on its enzymatic activity, resting membrane potential amplitude and duration of the action potential, reducing in this way contractility of the isolated rat ventricular right wall (Evangelista et al., 2010). Exposure to *Bothrops jararacussu* snake venom was found to cause an irreversible concentration-dependent contracture of the isolated rat atria which was confirmed by quantification of creatine kinase release and histological evidence of muscle damage, while pre-treatment with a sPLA₂ inhibitor abolished mentioned cardiotoxicity (Rodrigues et al., 2014). Also, myotoxicity and cardiotoxicity of the *Bungarus fasciatus* venom can be antagonized with compounds that inhibit sPLA₂ activity indicating that toxicity of the whole venom originates from sPLA₂ (Gomes et al., 2012).

Ethical statement

All procedures with animals were in strict accordance with the EU Directive 86/609/EEC, and Convention 123, the Directive 2010/63/EU. Ethics Committees of the Mostar University School of Medicine and Split University School of Medicine approved all experiments on animals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.toxicon.2016.09.001>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.toxicon.2016.09.001>.

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3.3 Znanstvena publikacija 3: Amoditin L je glavna kardiotoksična komponenta modrasovega strupa

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V nadaljevanju raziskav kardiotoksičnih učinkov modrasovega strupa v izoliranem in perfuziranem srcu podgane (poglavje 3.2) je sledila identifikacija kardiotoksične komponente v aktivni frakciji. V ta namen smo frakcijo C, ki je imela najbolj izrazit kardiotoksičen učinek (opisano v članku, predstavljenem v sekciji 3.2), ločili na štiri podfrakcije (C1, C2, C3 in C4) s pomočjo gelske filtracije. Le podfrakciji C1 in C2 sta inducirali podobne kardiotoksične učinke na izoliranem podganjem srcu kot celotna frakcija C, medtem ko frakciji C3 in C4 nista bili toksični.

Podfrakcije smo nato ločili na posamezne proteine s pomočjo RP-HPLC. Izolirane proteine smo identificirali s tandemsko MS analizo in določitvijo N-končnega aminokislinskega zaporedja po Edmanovi metodi. V vseh štirih podfrakcijah smo identificirali sledeče sPLA₂: AtnL₁, AtnL₂ in Atx A, B in/ali C. Le v podfrakcijah C1 in C2, ki sta izražali kardiotoksičnost, pa smo našli tudi proteina brez encimske aktivnosti, AtnL in CRISP.

AtnL je mikotoksični analog Atx, ki bi lahko povzročil lizo mišičnih celic srca [97]. Da bi preverili, ali je AtnL kardiotoksična komponenta strupa, smo izolirano podganje srce izpostavili čistemu AtnL in analizirali učinke na srčno funkcijo. AtnL je povzročil ireverzibilni kardiotoksični učinek (atrioventrikularno blokado), ne pa tudi srčnega zastoja. Poleg tega je povišan nivo biokemijskih markerjev (kreatin kinaza, laktat dehidrogenaza, aspartat aminotransferaza in troponin I) v iztoku iz srca pričal o poškodbi miokarda, podobno kot v primeru izpostavitve srca podfrakcijama C1 in C2. Vseh kardiotoksičnih učinkov, ki sta jih povzročili podfrakciji C1 in C2, nismo opazili, če smo v srce dovajali čisti AtnL, kar kaže, da za kardiotoksičnost v teh frakcijah ni odgovoren izključno AtnL. Skupna komponenta obema podfrakcijama, ki pa hkrati ni bila prisotna v netoksičnih podfrakcijah C3 in C4, je CRISP. Molekula CRISP je torej potencialni kardiotoksin v modrasovem strupu. Pokazali so, da določeni CRISPi iz KS zavirajo napetostno odvisne in od cikličnih nukleotidov odvisne ionske kanalčke v mišičnih celicah. Tak bi lahko bil tudi mehanizem delovanja modrasovega predstavnika.

Moj prispevek pri tej raziskavi je bilo delo, povezano s pripravo podfrakcij modrasovega strupa za poskuse na izoliranem podganjem srcu, analiza proteinske sestave podfrakcij, priprava vzorcev za MS in N-terminalno sekveniranje ter pomoč pri pisanju eksperimentalnih metod in pri analizi pridobljenih rezultatov.



Ammodytin L is the main cardiotoxic component of the *Vipera ammodytes ammodytes* venom



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ABSTRACT

Venom of the nose-horned viper (*V. a. ammodytes*) as also venoms of some related European vipers can induce also cardiotoxic effects in mammals. In this work we demonstrated that the protein in the *V. a. ammodytes* venom acting on heart is a myotoxic secreted phospholipase A₂ analogue ammodytin L (AtmL). In the isolated perfused rat heart AtmL induced significant and irreversible cardiotoxicity characterized by atrioventricular (AV) blockade. This venom protein induced appearance of high levels of creatine kinase, lactate dehydrogenase, aspartate aminotransferase and troponin I in the sinus effluent of the isolated heart, indicative for myocardial damage, which is obviously the primary cause of its cardiotoxic action. Gel filtration chromatography subfractions C1 and C2 of the *V. a. ammodytes* venom harboured most of the venom cardiotoxicity. As we showed, just these two subfractions contained also AtmL. Subfraction C1 in the final CF concentration 11.3 µg/mL (containing 3.1 µg/mL AtmL) induced a complete cardiac arrest while subfraction C2 in the final CF concentration 6.0 µg/mL (containing 0.8 µg/mL AtmL) and the pure AtmL (1.0 µg/mL) did not. Contrary to AtmL, subfraction C1 at 11.3 µg/mL was not able to induce the AV blockade. This exposed the only other cardiotoxic subfractions-specific venom protein, a cysteine-rich secretory protein (CRISP), as an additional venom component potentially involved in modulation of the heart activity. Cardiotoxicity reported in some cases of the adder (*V. berus*) venom and the asp viper (*V. aspis*) venom poisonings may be assigned to AtmL in these venoms.

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1. Introduction

The nose-horned viper, *Vipera ammodytes ammodytes* (*V. a. ammodytes*) is the largest and the most venomous European viper (Lukšić et al., 2006; Chippaux, 2012). Local and systemic clinical repercussions of the *V. a. ammodytes* envenomation in children and in adults have been well documented (Lukšić et al., 2006; Karabuvu et al., 2016a). *V. a. ammodytes* venom is a complex mixture of enzymatic and non-enzymatic components producing diverse pathophysiological effects (Georgieva et al., 2008; Križaj, 2011;

Sajević et al., 2013). Most frequent manifestations of poisoning by this venom are haematotoxicity and neurotoxicity (Lukšić et al., 2006; Karabuvu et al., 2016a). Cardiotoxicity has been only rarely reported, however, when emerged it caused life threatening instability of the patient due to severe myocardial dysfunctions, for example various rhythm abnormalities and even myocardial infarction (Aravanis et al., 1982; Lukšić et al., 2010). Toxic components of the *V. a. ammodytes* venom inducing neurological impairments have been thoroughly described from biochemical, molecular, immunological and other aspects (Križaj, 2011; Šribar et al., 2014). Also the venom components affecting haemostasis are well known (Sajević et al., 2011, 2013). On the contrary, molecular basis of the *V. a. ammodytes* venom cardiotoxicity is still unidentified. We documented previously a dose-dependent cardiotoxic effect of the standardized crude *V. a. ammodytes* venom and its gel filtration chromatography fraction C in a model of

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isolated rat heart (Lukić et al., 2008; Karabubu et al., 2016b).

Aiming to define, which component of the venom fraction C is responsible for the observed cardiotoxicity we split in this work the venom fraction C into four subfractions and tested each subfraction for its cardiotoxicity. We determined protein composition of subfractions and confirmed that ammodytin L (AtnL), a well-known myotoxic protein with a secreted phospholipase A₂ (sPLA₂) structure (Križaj et al., 1991) is the main cardiotoxic molecule of the V. a. ammodytes venom.

2. Materials and methods

2.1. Materials

Crude V. a. ammodytes venom was obtained from the Institute of Immunology Inc., Zagreb, Croatia. The venom was collected by "milking" snakes from regions of Krapina (70%) and Samobor (30%), dried at the ambient temperature, lyophilised and stored in dry, dark bottles at -20 °C until used. All other chemicals were of analytical and sequencing grade.

2.2. Methods

2.2.1. Reversed phase HPLC

The raw V. a. ammodytes venom was gel filtrated as described (Karabubu et al., 2016b) and the fraction C collected in four separate subfractions (C1 to C4). Each of the subfractions was further analysed on the HPLC (Hewlett-Packard Series 1100, USA) using the reversed phase C4 (RP-C4) column (Aquapore BU-300, 30 × 4.6 mm, 7 cm) equilibrated in 0.1% (v/v) trifluoroacetic acid in water (solution A) at flow rate of 1 mL/min 500 µL of each subfraction was diluted with solution A to 1 mL before the analysis. Proteins retained on the column were subsequently eluted with solution B (90% acetonitrile in solution A) at the same flow rate, using the following gradient: 0–20% in 5 min; 20–45% in 15 min; 45–60% in 5 min; 60–100% in 5 min. Elution of proteins was monitored by measuring the absorbance of the mobile phase at 215 nm (A₂₁₅). Chromatographic fractions were collected manually and dried in a vacuum concentrator (Savant, USA). Further analyses were performed at the ambient temperature.

2.2.2. SDS-polyacrylamide gel electrophoresis

Protein composition of the RP-HPLC subfractions C1 to C4 were analysed by SDS-PAGE using 12.5% (m/v) polyacrylamide gel under non-reducing conditions according to Laemmli (1970). Molecular mass standards were from Fermentas (Vilnius, Lithuania). Proteins were visualized by silver staining.

2.2.3. Concentrations of venom proteins used in experiments on rat heart

Lukić et al. (2008) reported cardiotoxic effects in isolated rat heart using the final coronary flow (CF) venom protein concentrations of 7.5, 22.5 and 37.5 µg/mL, and Karabubu et al. (2016b) registered cardiotoxicity of the venom gel filtration fraction C in equivalent doses. With an estimation that the subfraction C1 contains 30% (m/m) of total protein content of the fraction C, we used in our testing the final CF concentrations of 2.3, 6.8 and 11.3 µg/mL of this subfraction. The subfraction C2 was estimated to consist of 16% (m/m) of all proteins present in the fraction C so it was tested in doses 1.2, 3.6 and 6.0 µg/mL. As approximated, the subfraction C3 harboured 21% (m/m) of the fraction C proteins. It was probed therefore in concentrations 1.6, 4.7 and 7.9 µg/mL. Finally, the subfraction C4, representing the remaining 33% (m/m) fraction C proteins, was applied to the isolated rat heart in the final CF concentrations of 2.5, 7.4 and 12.4 µg/mL.

AtnL was prepared as described (Gubenšek et al., 1980; Thoun et al., 1982; Križaj et al., 1991) and dissolved in water at 100 µg/mL. Each isolated rat heart was exposed to AtnL at the final CF concentration of 1.0 µg/mL.

2.2.4. Rat heart preparation and recording of heart functioning

Cardiac experiments were done using Langendorff model of the isolated perfused rat heart as previously described (Karabubu et al., 2016b). The following heart parameters were recorded: Heart rate (HR – beats/min) was measured as atrial beats in ex vivo hearts. Atrioventricular (AV) conduction time (ms) was determined as the period between the right atrial beat and the right ventricular conus beat. This parameter was used also to determine the prolongation of AV conduction time in order to define AV blockade, which was registered as a condition when one or more, but not all of the atrial impulses were unable to pulse to the ventricles. Ventricular tachycardia (VT – N and %) was defined as four or more consecutive waveforms of ventricular activity, and a faster ventricular than atrial rate. Ventricular fibrillation (VF – N and %) was noted by the presence of irregular action in the ventricular electrocardiogram, and by the absence of pressure generation by the left ventricle. No electrical and mechanical activity of the myocardial muscle was considered as asystolic cardiac arrest (ASYS – N and %). Coronary flow (CF – mL/min) and the pressures in the left ventricle (mmHg) (developed left ventricular pressure – devLVP, diastolic left ventricular pressure – dLVP and systolic left ventricular pressure – sLVP) were measured as previously described (Karabubu et al., 2016b). Biochemical markers of heart injury, creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and troponin I, were measured in the 3rd min after the heart was exposed to a venom sample by taking blood sample from the sinus effluent, according to the methods presented in Lukić et al. (2008).

2.2.5. Experimental protocol

We used the same experimental setup as described in Karabubu et al. (2016b). Biochemical markers of myocardial damage were measured following 3 min of exposure of the heart to a venom sample, when cardiotoxic effect has already been fully developed. All handling and procedures with used animals were in strict accordance with the EU Directive 86/609/EEC, and Convention 123, the Directive 2010/63/EU. Ethics Committees of the Mostar University School of Medicine and Split University School of Medicine approved all experiments on animals.

2.2.6. Statistical analysis

Qualitative variables are presented as counts (N) and percentages, and were tested using χ^2 -test. Quantitative data are expressed as mean ± standard deviation (mean ± SD), and were tested with analysis of variance (ANOVA) and Kruskal-Wallis test, followed by post-hoc Bonferroni or Tukey's multiple comparisons. Data were calculated using SPSS Statistics (version 14.0, SPSS INC., Chicago, Illinois, USA). P value < 0.01 was considered as statistically significant.

2.2.7. N-terminal protein sequencing

N-terminal amino acid sequencing of proteins was accomplished using a Procise 492A Automated Sequencing System (Applied Biosystems, USA).

2.2.8. Mass spectrometry

Proteins have been analysed using electro spray ionization quadrupole time-of-flight tandem mass spectrometry (ESI Q-TOF MS/MS; Waters Micromass, UK) as described (Sosić et al., 2016).

3. Results

3.1. Preparation of the venom subfractions

Using gel filtration chromatography, the raw *V. a. ammodytes* venom was separated in four major fractions, A to D. Most of the venom cardiotoxicity was concentrated in fraction C (Karabuda et al., 2016b). To narrow a complement of venom proteins which activity is potentially directed to heart, we subdivided fraction C by collecting it into four separate test tubes to obtain subfractions C1 to C4.

3.2. Effects of the venom subfractions on the rat heart ex vivo

Venom subfractions C1, C2, C3 and C4 were tested for their effects on HR, CF, sLVP, devLVP and dLVP in the system of the isolated perfused rat heart (Langendorff model). The most prominent change in HR was produced by the subfraction C1 at its highest final CF concentration 11.3 µg/mL (Table 1, Suppl. Fig. 1). All hearts exposed to such dose of the subfraction C1 ($N = 10$) suffered irreversible cardiac arrest already in the 3rd min of envenomation period, supported with an obvious statistical significance (1.0 ± 0.0 beats/min vs. 291.2 ± 3.9 beats/min at the time 0, $P < 0.01$; Table 1). Also, all 10 hearts that stopped beating ended in asystolia with VT preceding VF. AV blockade was not registered while testing subfraction C1. In accordance with electrophysiological changes, parameters of contractile function changed in conformity with the

irreversible myocardial contracture. Therefore, CF significantly decreased in the 3rd min of exposure to the highest dose of subfraction C1 (4.3 ± 0.2 mL/min vs. 11.2 ± 0.2 mL/min at the time 0, $P < 0.01$), and also in the 13th min, this is 3 min after stopping the venom sample delivery (3.6 ± 0.4 mL/min vs. 11.2 ± 0.2 mL/min at the time 0, $P < 0.01$; Table 1). Decrease of devLVP from 116.4 ± 1.2 mmHg at the beginning of the experiment to 1.2 ± 0.4 mmHg in its 3rd min was accompanied by an increase of dLVP from 0 mmHg to 59.2 ± 1.2 mmHg in the same time span ($P < 0.01$; Table 1). At two lower final CF concentrations tested, 2.3 and 6.8 µg/mL, subfraction C1 did not induce any significant toxic effect on the rat heart (Tables 1 and 2, Suppl. Fig. 1).

Subfraction C2 affected the rat heart in a similar way as subfraction C1. Cardiotoxicity of this subfraction was registered only at the highest final CF concentration tested (6.0 µg/mL), at which it did not induce cardiac arrest (Tables 1 and 2, Suppl. Fig. 1). It was manifested as irreversible and statistically significant decrease of HR in the 3rd and 13th min (235.0 ± 6.7 beats/min and 233.7 ± 5.6 beats/min, respectively, vs. 292.1 ± 3.7 beats/min at the beginning of the experiment ($P < 0.01$; Table 1), and significant lowering of CF in the 13th min in comparison to the time 0 (8.0 ± 0.1 mL/min vs. 11.0 ± 0.2 mL/min, $P < 0.01$; Table 1). No rhythm disturbances were noticed during experiments with subfraction C2. Mentioned cardiotoxic relations of heart contracture were in accordance with statistically significant decrease of the devLVP and insignificant increase of dLVP (Table 1, Suppl. Fig. 1).

Venom subfractions C3 and C4 did not affect any of the cardiac

Table 1
Cardiac parameters measured in the Langendorff model of the isolated perfused rat heart. Rat hearts were perfused with three different doses of subfractions C1 and C2 of the *V. a. ammodytes* venom gel filtration chromatography fraction C and the solution of AtnL. Heart rate (HR), coronary flow (CF), systolic left ventricular pressure (sLVP), developed left ventricular pressure (devLVP) and diastolic left ventricular pressure (dLVP) were registered in each experiment in three different time points: at the beginning of the experiment, after 3 min of the sample delivery and 3 min after the stop of the sample delivery. All values are expressed as mean $\pm$ SD of ten independent measurements, and tested using ANOVA.

Subfraction [µg/mL]	Time [min]	Cardiac parameters [mean $\pm$ SD]				
		HR [beats/min]	CF [mL/min]	sLVP [mmHg]	devLVP [mmHg]	dLVP [mmHg]
Control		289.6 $\pm$ 1.6	11.1 $\pm$ 0.1	116.0 $\pm$ 2.3	116.0 $\pm$ 2.3	0
	0	288.8 $\pm$ 1.4	11.3 $\pm$ 0.2	116.3 $\pm$ 2.4	116.3 $\pm$ 2.4	0
	3	287.5 $\pm$ 1.4	11.2 $\pm$ 0.1	115.6 $\pm$ 1.5	115.6 $\pm$ 1.5	0
C1 [2.3]		286.2 $\pm$ 1.3	11.1 $\pm$ 0.1	115.1 $\pm$ 2.0	115.1 $\pm$ 2.0	0
	0	296.4 $\pm$ 2.1	11.2 $\pm$ 0.2	116.6 $\pm$ 2.6	116.6 $\pm$ 2.6	0
	3	293.2 $\pm$ 2.7	11.2 $\pm$ 0.1	115.1 $\pm$ 1.2	115.1 $\pm$ 1.2	0
C1 [6.8]		289.3 $\pm$ 2.5	11.1 $\pm$ 0.1	114.5 $\pm$ 1.4	114.5 $\pm$ 1.4	0
	0	291.2 $\pm$ 3.9	11.2 $\pm$ 0.2	116.4 $\pm$ 1.2	116.4 $\pm$ 1.2	0
	3	1.0 $\pm$ 0.0 [†]	4.3 $\pm$ 0.2 [†]	60.4 $\pm$ 1.4 [†]	1.2 $\pm$ 0.4 [†]	59.2 $\pm$ 1.2 [†]
C1 [11.3]		293.1 $\pm$ 2.5	11.1 $\pm$ 0.1	116.0 $\pm$ 1.8	116.0 $\pm$ 1.8	0
	0	291.2 $\pm$ 3.9	11.2 $\pm$ 0.2	116.4 $\pm$ 1.2	116.4 $\pm$ 1.2	0
	3	1.0 $\pm$ 0.0 [†]	3.6 $\pm$ 0.4 [†]	62.3 $\pm$ 1.0 [†]	1.0 $\pm$ 0.0 [†]	53.2 $\pm$ 1.1 [†]
Control		291.2 $\pm$ 2.7	11.2 $\pm$ 0.2	116.2 $\pm$ 1.2	116.2 $\pm$ 1.2	0
	0	288.8 $\pm$ 2.4	11.2 $\pm$ 0.1	115.8 $\pm$ 1.3	115.8 $\pm$ 1.3	0
	3	286.5 $\pm$ 1.9	11.0 $\pm$ 0.1	115.3 $\pm$ 1.2	115.3 $\pm$ 1.2	0
C2 [1.2]		289.2 $\pm$ 2.3	11.0 $\pm$ 0.3	116.9 $\pm$ 2.3	116.9 $\pm$ 2.3	0
	0	287.9 $\pm$ 2.1	10.9 $\pm$ 0.1	116.6 $\pm$ 2.1	116.6 $\pm$ 2.1	0
	3	286.5 $\pm$ 2.3	10.9 $\pm$ 0.2	115.3 $\pm$ 2.0	115.3 $\pm$ 2.0	0
C2 [3.6]		292.1 $\pm$ 3.7	11.1 $\pm$ 0.2	116.7 $\pm$ 1.2	116.7 $\pm$ 1.2	0
	0	235.0 $\pm$ 6.7 [†]	9.3 $\pm$ 1.0	94.3 $\pm$ 2.1 [†]	91.2 $\pm$ 1.2 [†]	3.1 $\pm$ 0.6
	3	233.7 $\pm$ 5.6 [†]	8.0 $\pm$ 0.1 [†]	94.0 $\pm$ 1.3 [†]	91.1 $\pm$ 1.1 [†]	2.9 $\pm$ 0.4
C2 [6.0]		292.6 $\pm$ 2.3	11.1 $\pm$ 0.2	116.2 $\pm$ 1.7	116.2 $\pm$ 1.7	0
	0	292.1 $\pm$ 3.7	11.0 $\pm$ 0.1	115.8 $\pm$ 1.3	115.8 $\pm$ 1.3	0
	3	221.0 $\pm$ 4.3 [†]	8.1 $\pm$ 0.2 [†]	90.1 $\pm$ 1.6 [†]	77.8 $\pm$ 1.5 [†]	12.3 $\pm$ 0.4 [†]
Control		219.4 $\pm$ 5.1 [†]	7.7 $\pm$ 0.1 [†]	89.2 $\pm$ 1.2 [†]	76.2 $\pm$ 1.8 [†]	13.1 $\pm$ 0.6 [†]
	0	292.1 $\pm$ 3.7	11.0 $\pm$ 0.1	115.8 $\pm$ 1.3	115.8 $\pm$ 1.3	0
	3	221.0 $\pm$ 4.3 [†]	8.1 $\pm$ 0.2 [†]	90.1 $\pm$ 1.6 [†]	77.8 $\pm$ 1.5 [†]	12.3 $\pm$ 0.4 [†]
AtnL [1.0]		219.4 $\pm$ 5.1 [†]	7.7 $\pm$ 0.1 [†]	89.2 $\pm$ 1.2 [†]	76.2 $\pm$ 1.8 [†]	13.1 $\pm$ 0.6 [†]
	0	292.1 $\pm$ 3.7	11.0 $\pm$ 0.1	115.8 $\pm$ 1.3	115.8 $\pm$ 1.3	0
	3	221.0 $\pm$ 4.3 [†]	8.1 $\pm$ 0.2 [†]	90.1 $\pm$ 1.6 [†]	77.8 $\pm$ 1.5 [†]	12.3 $\pm$ 0.4 [†]

[†] $P < 0.01$ vs. control cardiac parameters.

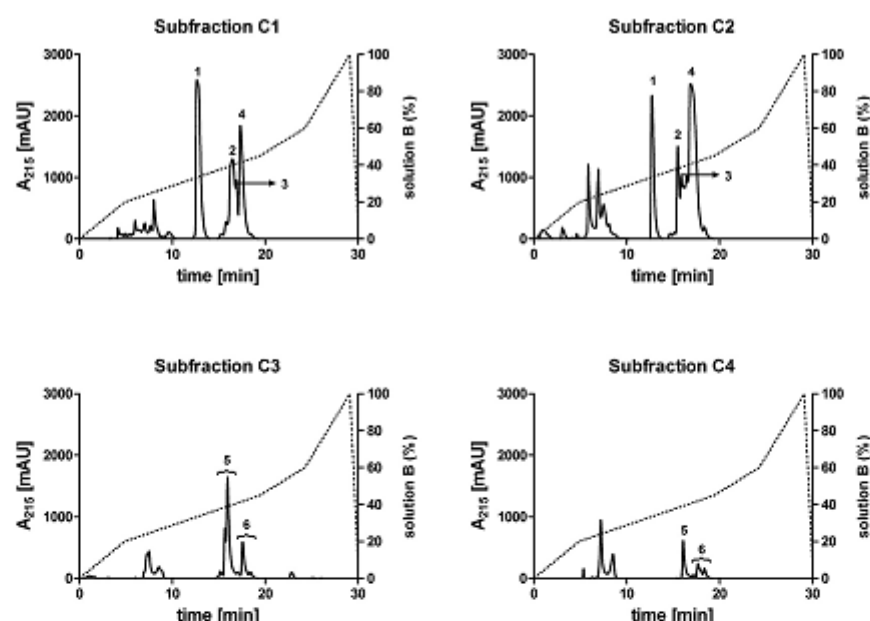


Fig. 1. RP-HPLC separation of *V. a. ammodytes* subfractions C1 to C4. Subfractions C1 to C4, obtained by gel filtration chromatography of the raw *V. a. ammodytes* venom, were applied on Aquapore BU-300 (30 × 4.6 mm, 7 cm) column equilibrated in 0.1% (v/v) trifluoroacetic acid in water (solution A). Elution of the column-bound molecules was achieved by gradually increasing the concentration of solution B (90% (v/v) acetonitrile in solution A) as designated. Analyses were performed at the flow rate of 1 mL/min. The absorbance was monitored at 215 nm (A_{215}). Numbered peaks were collected and dried before structural analysis.

Table 2

Biochemical markers of myocardial damage in the Langendorff model of the isolated perfused rat heart. Rat hearts were perfused with three different doses of subfractions C1 – C4 of the *V. a. ammodytes* venom gel filtration chromatography fraction C and the solution of AtmL. Creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and troponin I were measured in each experiment in the sinus effluent of the isolated rat heart after 3 min of the sample delivery. All values are expressed as means ± SD of ten independent measurements, and tested using ANOVA.

Subfraction	Concentration [μ g/mL]	Biochemical parameters [mean ± SD]			
		CK [IU/L]	LDH [IU/L]	AST [IU/L]	Troponin I [μ g/L]
Control		2.11 ± 0.65	0.24 ± 0.16	3.18 ± 0.88	0.01 ± 0.01
C1	2.3	2.22 ± 0.98	0.77 ± 0.15	3.23 ± 1.11	0.02 ± 0.01
	6.8	2.43 ± 0.88	0.91 ± 0.34	6.65 ± 0.99	0.07 ± 0.02
	11.3	79.14 ± 2.02 [†]	198.08 ± 1.88 [†]	399.78 ± 4.43 [†]	7.78 ± 0.04 [†]
C2		2.22 ± 0.51	0.33 ± 0.20	3.94 ± 1.11	0.02 ± 0.01
	1.2	2.25 ± 0.62	1.27 ± 1.34	3.88 ± 1.28	0.03 ± 0.01
	3.6	3.17 ± 1.15	1.91 ± 0.69	5.03 ± 2.32	0.07 ± 0.03
C3	6.0	14.68 ± 2.10 [†]	19.71 ± 1.24 [†]	178.34 ± 2.78 [†]	5.01 ± 0.02 [†]
		2.36 ± 0.76	0.54 ± 0.19	3.76 ± 0.68	0.03 ± 0.01
C4	1.6	2.22 ± 0.97	0.79 ± 0.13	3.77 ± 1.15	0.03 ± 0.01
	4.7	3.46 ± 1.01	0.99 ± 0.43	4.43 ± 0.97	0.02 ± 0.01
	7.9	3.73 ± 0.99	1.21 ± 0.37	4.73 ± 1.01	0.01 ± 0.01
Control AtmL		2.88 ± 0.27	0.44 ± 0.16	3.79 ± 0.74	0.03 ± 0.01
	2.5	2.61 ± 0.62	0.86 ± 0.14	3.98 ± 1.19	0.03 ± 0.01
	7.4	2.46 ± 1.11	1.13 ± 0.55	4.39 ± 1.10	0.02 ± 0.01
Control		2.73 ± 1.13	1.26 ± 0.26	4.53 ± 1.13	0.01 ± 0.01
	12.4	2.76 ± 0.23	0.45 ± 0.11	3.26 ± 0.66	0.02 ± 0.01
	1.0	15.37 ± 2.13 [†]	24.23 ± 2.16 [†]	173.64 ± 4.26 [†]	5.78 ± 0.04 [†]

[†]P < 0.01 vs. control cardiac parameters.

parameters measured (Suppl. Table 1).

The extent of myocardial damage has been evaluated by measuring concentrations of enzymes, CK, LDH and AST, and troponin I in the sinus effluent of the isolated rat heart after 3 min of exposure of the heart to venom samples, at the point when cardiotoxic effect has already been fully manifested. While venom subfractions C3 and C4 did not influence these biochemical indicators, subfractions C1 and C2 drastically increased their concentrations in the rat heart perfusate (Table 2). At the highest concentration tested (11.3 µg/mL), subfraction C1 induced 38-fold increase in CK activity, 825-fold increase in LDH activity, 126-fold increase in AST activity and 778-fold increase in troponin I concentration in the sinus effluent (Table 2). The highest concentration of subfraction C2 tested was 6.0 µg/mL, and this induced 7-fold increase in CK activity, 60-fold increase in LDH activity, 45-fold increase in AST activity and 251-fold increase in troponin I content in the perfusate (Table 2).

Presented results clearly show that cardiotoxicity of the *V. a. ammodytes* venom is confined to subfractions C1 and C2.

3.3. Biochemical characterisation of the venom subfractions

To get an idea about possible cardiotoxic components in subfractions C1 and C2 of the *V. a. ammodytes* venom, each subfraction was chromatographed on the RP-HPLC C4 column and the separated polypeptides structurally characterized. Peptides of 800–1200 Da eluted from the column during the first 10 min of analysis then proteins followed (Fig. 1). Molecular identity of proteins was specified by Edman sequencing and MS (Table 3). In all four subfractions different sPLA₂s, ammodytoxins (Atxs) and ammodytins (Atns) (Krizaj et al., 2011) were detected. An sPLA₂-like protein AtnL (Krizaj et al., 1991) and a cysteine-rich secretory protein (CRISP) were discovered exclusively in both cardiotoxic subfractions. Due to its well established myotoxic activity, AtnL appeared as the main candidate protein to act adversely on heart. From RP-HPLC chromatograms in Fig. 1 we estimated that AtnL represented 28% (m/m) of proteins in subfraction C1 and 13% (m/m) of proteins in subfraction C2. On this basis we concluded that probing the pure AtnL on rat heart at the final CF concentration 1.0 µg/mL should undoubtedly reveal whether the molecule is cardiotoxic or not.

3.4. Effects of the AtnL on the rat heart ex vivo

To verify our assumption of AtnL as the venom component with cardiotoxic activity we measured the effects of the pure AtnL on the isolated rat heart (Table 1, Suppl. Fig. 1). Perfusion of the hearts with the solution containing 1.0 µg/mL AtnL significantly lowered the HR observed in the 3rd and 13th min of the experiment (221.0 ± 4.3

beats/min and 219.4 ± 5.1 beats/min, respectively) comparing to the initial value (292.1 ± 3.7 beats/min, $P < 0.01$; Table 1). Also the CF values considerably decreased from initial 11.0 ± 0.1 mL/min to 8.1 ± 0.2 and 7.7 ± 0.1 mL/min in the 3rd and 13th min of the experiment ($P < 0.01$; Table 1). The same happened to sLVP and devLVP. The first reduced from the initial value of 115.8 ± 1.3 mmHg to 90.1 ± 1.6 mmHg in the 3rd min and 89.2 ± 1.2 mmHg in the 13th min of experiment, while the latter dropped from 115.8 ± 1.3 mmHg at the time 0 to 77.8 ± 1.5 mmHg in the 3rd min and 76.2 ± 1.8 mmHg in the 13th min of experiment. Oppositely, dLVP went up from 0 mmHg (min 0) to 12.3 ± 0.4 mmHg (min 3) and 13.1 ± 0.6 mmHg (min 13). Any of the observed cardiotoxic effects did not revert following cessation of the delivery of AtnL meaning that the damage caused by AtnL to the heart was irreversible (Suppl. Fig. 1). AtnL induced AV blockade in all tested hearts ($N = 10$), but no other type of arrhythmias. AV blockade occurred always about 3 min from the start of the AtnL delivery to the heart and lasted during the whole experiment. No cardiac arrest was registered at 1.0 µg/mL AtnL.

4. Discussion

As we reported, the large majority of cardiotoxicity of the *V. a. ammodytes* venom was concentrated in its gel filtration chromatography fraction C (Karabuvu et al., 2016b). Its effects on the heart were similar to those inflicted by the raw venom (Lukšić et al., 2008). Fraction C caused considerable decrease of HR and CF, together with the increase of dLVP joined with the decrease of sLVP and devLVP. In this work, the venom fraction C was split by gel filtration chromatography into four subfractions, C1 to C4, and tested in the Langendorff model of the isolated perfused rat heart for their influence on the following cardiac parameters, HR, CF, sLVP, devLVP and dLVP. Subfractions C1 and C2 affected these heart parameters in a very similar way as the venom fraction C (Table 1 and Suppl. Fig. 1; Karabuvu et al., 2016b) while subfractions C3 and C4 were ineffective even at the highest doses (Suppl. Table 1). Subfraction C1, at the highest dose tested (11.3 µg/mL), induced irreversible asystolic arrest of rat hearts. Cardiac arrest did not occur at a lower dose of subfraction C1 (6.8 µg/mL) as also not at the highest dose of subfraction C2 tested (6.0 µg/mL) suggesting its dependence on the concentration of the cardiotoxic component(s). To identify proteins present in the venom subfractions, each subfraction was chromatographed on the RP-HPLC (Fig. 1) and the separated components subjected to Edman degradation and ESI Q-TOF-MS/MS analysis. AtnL, a myotoxic sPLA₂-like protein (Krizaj et al., 1991), and a CRISP molecule (Yamazaki and Morita, 2004), were exclusively found only in cardiotoxic subfractions C1 and C2 (Table 3), which exposed these two proteins as possible heart-targeting components of the venom. In all four subfractions mixture of different sPLA₂s, AtnL₁, AtnL₂ and Atxs, was also detected (Fig. 1 and Table 3) but, as demonstrated by the absence of any effect of subfractions C3 and C4 on the rat heart, these molecules were not cardiotoxic.

Increased levels of biochemical markers, CK, LDH, AST and troponin I, in the sinus effluent of the isolated rat heart following exposure of the heart to cardiotoxic subfractions C1 and C2 revealed that injury of the heart took place (Table 2). This suggested myotoxic AtnL as the most plausible cardiotoxic substance in the venom. Strongly supporting such assumption, the extent of the myocardial damage reflected in the level of biochemical markers in the heart effluent corresponded to the concentration of AtnL in the heart perfusate. We ultimately confirmed that AtnL acts cardiotoxically by exposing the rat heart to the pure AtnL obtaining the same main effects on the heart as with subfractions C1 and C2 (Tables 1 and 2, Suppl. Fig. 1). Atxs, enzymatically active paralogs of

Table 3
Structural identification of proteins in subfractions C1 to C4. Dry RP-HPLC fractions (Fig. 1) were dissolved in 20% (v/v) acetonitrile. Molecular masses of proteins in fractions were determined using ESI Q-TOF MS/MS while their N-terminal amino acid sequences by automated Edman degradation. Abbreviations: ammodytin I [I]/I2/I, AtnL1/I2/I; ammodytin A [A]/A2/A, AtxA1/A2/A; cysteine-rich secretory protein, CRISP.

Fraction	Edman sequencing	M + H ⁺ [Da]	Protein
1	SVIEF	13858	AtnL
2	NLYQF	13628 + 13558	AtnL ₁
3	SVDFDSEP	24674	CRISP
4	HLNQFGDM	13700 + 13683	AtnL ₁
	SLL	13746	AtxC
5	NLYQF	13623 + 13558	AtnL ₂
6	SLL	13779 + 13746	AtxA1/A2/A
		13780 + 13778	AtxA1/A2/A

AtnL, which we were not able to lyse muscle cells (Križaj et al., 1991) were also not able to affect the heart as we demonstrated (Suppl. Table 1, Fig. 1 and Table 3). Cardiotoxicity of AtnL is most likely the consequence of its myotoxicity. A myotoxic sPLA₂ molecule, Lys49 sPLA₂ protein from the venom of a pit viper *Agkistrodon p. piscivorus*, has already been indicated as being cardiotoxic (Dhillon et al., 1987). It is also known that venoms of snakes from the genus *Bothrops* contain high concentrations of sPLA₂ myotoxins and that cardiotoxicity is a common cause of death after *Bothrops* snakebite (Sifuentes et al., 2008).

In the final CF concentration 1.0 µg/mL, AtnL induced in the rat heart all of the effects that were induced also by the *V. a. ammodytes* venom subfraction C1, with one exception. The AV blockade was recorded in the case of pure AtnL but not also in the case of the venom subfraction C1. This may indicate that besides AtnL additional component exists in the nose-horned viper venom that modulates the heart response. Candidate molecule to be further explored in this direction is obviously a CRISP molecule that was also present just in the two cardiotoxic venom subfractions (Fig. 1 and Table 3). Certain snake venom CRISPs have been demonstrated to inhibit voltage-gated or cyclic nucleotide-gated ion channels in muscle cells (Yamazaki et al., 2003; Yamazaki and Morita, 2004; Ramazanov et al., 2009) and such function may be anticipated also for the *V. a. ammodytes* venom CRISP.

There are six endemic species of vipers in Europe. Although rarely, cardiotoxicity has been undoubtedly recorded in patients poisoned by the nose-horned viper (*V. a. ammodytes*) venom (Aravanis et al., 1982; Lukšić et al., 2010), the adder (*V. berus*) venom (Chadha et al., 1968; Moore, 1988; Magdalan et al., 2010; Czajka et al., 2013; Hønge et al., 2015) and the asp viper (*V. aspis*) venom (De Haro et al., 2009).

The *V. berus* venom can induce electrocardiographic T wave inversion or flattening, ST elevation, second degree AV block, brady/tachyarrhythmias, atrial fibrillation and myocardial infarction (Moore, 1988; Magdalan et al., 2010; Czajka et al., 2013; Hønge et al., 2015). Arguments for a direct toxic effect of the adder venom on the myocardium have been also presented in the case of a 14 years old infant (Chadha et al., 1968). Existence of AtnL in the *V. berus* venom has been confirmed (Latinović et al., 2016) so we suggest that AtnL is mainly responsible for cardiotoxic effects also in the case of the adder venom poisoning.

Direct intravascular injection of the *V. aspis* venom induced massive myocardial injury and led to a rapid death of a 45-year-old man (De Haro et al., 2009). The expression of AtnL in this venom has not been confirmed yet but it is possible that, similarly as in the case of neurotoxic Atns, the myotoxic *V. aspis* snake population appeared as a result of hybridization between *V. aspis* and *V. ammodytes* species (Ferquel et al., 2007).

Cardiotoxicity is usually an irrelevant effect at envenomation of humans by one of the European vipers. Exceptional in this respect can be the cases where snake injects its venom directly into the bloodstream (De Haro et al., 2009) or in the vicinity of the heart of the victim. Exceptional can also be envenomations of young people as snakes do not adjust the volume of venom ejected to the body mass of a prey or predator allowing cardiotoxic components to reach the effective concentrations in the blood (Lukšić et al., 2010). Cardiotoxicity is probably more regular way to subdue rodents, natural prey of vipers. We demonstrated here that the main cardiotoxic molecule in *V. a. ammodytes* venom, as likely also in venoms of the other European vipers, is the myotoxic sPLA₂-like protein AtnL.

Ethical statement

All procedures with animals were in strict accordance with the

EU Directive 86/609/EEC, and Convention 123, the Directive 2010/63/EU. Ethics Committees of the Mostar University School of Medicine and Split University School of Medicine approved all experiments on animals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.toxicon.2017.10.003>.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.toxicon.2017.10.003>.

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3.4 Znanstvena publikacija 4: Strukturna in biokemijska karakterizacija molekule VaF1, fibrinogenolizne metaloproteinaze P-IIIa iz modrasovega strupa

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Modrasov strup je vir bioloških molekul z izrazitim vplivom na srčno-žilni sistem. V primerjavi z gadovim strupom ima večji nabor hemostatsko aktivnih proteinov, kar je podrobno opisano v znanstveni publikaciji, predstavljeni v sekciji 3.1. Molekule lahko delujejo prokoagulantno, tako da aktivirajo FX, protrombin in/ali fibrinogen, oziroma antikoagulantno, tako da cepijo fibrin(ogena), inhibirajo koagulacijske faktorje ali učinkujejo na proces agregacije trombocitov. Širok spekter omenjenih aktivnosti imajo MP iz KS, ki jih delimo na tri razrede: P-I, P-II in P-III, glede na njihovo domensko sestavo.

V tej znanstveni publikaciji smo podrobneje opisali osnovne biokemijske značilnosti nove fibrinogenolizne MP iz modrasovega strupa, ki smo jo poimenovali *Vipera ammodytes* Fibrinogenaza 1 (VaF1).

S pomočjo začetnih oligonukleotidov, pripravljenih na osnovi ohranjenih 5' in 3' neprevajajočih se regij mRNA za P-III MP, smo v cDNA knjižnici modrasove strupne žleze našli celotno aminokislinsko zaporedje VaF1. Z MS analizo smo določili 34 % aminokislinskega zaporedja zrele oblike proteina. VaF1 ima MP, disintegrinu podobno in s cisteinom bogato domeno ter nima prostega Cys. Omenjene karakteristike jo določajo kot MP razreda P-III. Poleg tega smo določili biološke tarče oziroma substrate VaF1, komponente hemostatskega sistema.

VaF1 ima molekulsko maso 49,735 kDa in je N-glikoziliran protein z dvema potencialnima mestoma za N-glikozilacijo. Skladno s tem se pojavlja v več izooblikah z izoelektričnimi točkami okrog pH 5,8. VaF1 specifično cepi α -verigo fibrinogena. Poleg tega cepi tudi proteine koagulacijske kaskade, kot sta FX in protrombin, ter plazminogen, vendar tako, da jih ne aktivira. VaF1 šibko hidrolizira proteine zunajceličnega matriksa in bazalne membrane (kolagen IV, nidogen in fibronektin), vendar ne povzroči hemoragije v miškah *in vivo*. Kljub prisotnosti kolagen-vezavnega aminokislinskega zaporedja v domeni D, VaF1 nima učinka na agregacijo trombocitov.

Kot encimsko aktiven protein, čigar delovanje preprečuje normalno strjevanje krvi, je bilo treba preveriti, če ga prepozna anti-*Vaa*. Anti-*Vaa* kot tudi anti-H ne prepoznata VaF1. Anti-H je prepoznal protein VaF1 le v čisti obliki, ne pa tudi v surovem strupu. Toksini, ki jih protistrup ne prepozna, lahko povzročijo zaplete pri zdravljenju zastrupitve s seroterapijo. Z identifikacijo takih toksinov, kot je tudi VaF1, lahko torej izboljšamo tak način zdravljenja.

S tem delom smo potrdili tudi našo drugo hipotezo, ki trdi, da modrasov strup vsebuje α -fibrinogenaze. VaF1, ki s cepitvijo α -verige fibrinogena deluje antikoagulantno, je primerna terapevtska učinkovina za defibrinogenacijo.

Moj prispevek pri tem znanstvenem delu je bila izvedba izoelektričnega fokusiranja vzorca, analiza cepitve fibrinogena in imunološke analize. Poleg tega sem pomagala pri pisanju eksperimentalnih metod in pri analizi pridobljenih rezultatov ter pripravi slik.

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Research paper

Structural and biochemical characterisation of VaF1, a P-IIIa fibrinogenolytic metalloproteinase from *Vipera ammodytes ammodytes* venom

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ABSTRACT

A high molecular mass metalloproteinase with α -fibrinogenolytic activity, termed VaF1, was purified from nose-horned viper (*Vipera ammodytes ammodytes*) venom. Subcutaneous injection of 9 μ g of VaF1 did not induce bleeding in rats. Nevertheless, *in vitro* it degraded collagen IV, nidogen and fibronectin, components of the extracellular matrix, although with low efficacy and narrow specificity. VaF1 would be expected to exert anti-coagulant action, due to its hydrolysis of fibrinogen, factor X, prothrombin and plasminogen, plasma proteins involved in blood coagulation. The enzyme is a single-chain glycoprotein with a molecular mass of 49.7 kDa, as determined by mass spectrometry, and multiple isoelectric points centred at pH 5.8. The complete amino acid sequence of the precursor of VaF1 was deduced by cloning and sequencing its cDNA. Composed of metalloproteinase, disintegrin-like and cysteine-rich domains, VaF1 is a typical P-IIIa subclass snake venom metalloproteinase. Although it possesses a collagen-binding sequence in its disintegrin-like domain, VaF1 displayed no effect on collagen-induced platelet aggregation *in vitro*. Two consensus N-glycosylation sites are present in the sequence of VaF1, however, the extent of its glycosylation is low, only 5.2% of the total molecular mass. Interestingly, in standard experimental conditions VaF1 is not recognised by antiserum against the whole venom, so it can contribute to post-serotherapy complications, such as ineffective blood coagulation, in the envenomed patient.

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1. Introduction

Snake venom (SV) is a mixture of various pharmacologically active proteins and peptides that affect different physiological systems, mostly nervous, muscular, and cardiovascular. Many of these components are highly specific for a variety of molecular targets and thus constitute potential therapeutic and diagnostic agents [1–4]. Viperid snake venoms are particularly rich in proteins that disturb the haemostatic system, acting as pro-coagulants that activate coagulation factor X (FX), prothrombin and fibrinogen, or as anti-coagulants, lysing fibrin(ogen), inhibiting coagulation factors and interfering with platelet aggregation [4]. Of these proteins,

Abbreviations: ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin type-1 motif; BM, basal membrane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Cys-rich, cysteine-rich; dis-like, disintegrin-like; ECM, extracellular matrix; FX, factor X; GP, platelet glycoprotein; MES, 2-[(N-morpholino)ethanesulfonic acid]; MP, metalloproteinase; PNGF, peptide N-glycosidase F; SP, serine protease; SV, snake venom; SVMP, snake venom metalloproteinase; Vaa, *Vipera ammodytes ammodytes*; VaF1, *Vipera ammodytes* fibrinogenase 1; VaH, *Vipera ammodytes* hemorrhagin.

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metalloproteinases (MPs) have the broadest spectrum of activities, a consequence of their modular structure. Snake venom MPs (SVMs) are Zn^{2+} -dependent enzymes that are synthesised in venom glands as multidomain, inactive zymogens and subsequently activated by the cysteine-switch (Cys-switch) mechanism, common to SV- and matrix MPs [5,6]. According to their domain organisation and post-translational processing, three major classes of SVMs exist [7]. In the P-I class, the pro-domain is followed by a catalytic MP domain. Two motifs characterise the catalytic site in the MP domain, the Zn^{2+} -binding motif (HEXXHXXGXXH) and the methionine-turn (C-I-M-S-X) [8]. P-II SVMs are composed of the MP domain and a disintegrin domain, and serve as precursors of disintegrins. In the P-III class SVMs, the MP domain is followed by a disintegrin-like (dislike) and a cysteine-rich (Cys-rich) domain. Depending on the type of maturation of the P-II and P-III class SVMs, which can be dimerisation, proteolytic processing and inter-molecular disulphide bond formation, these molecules are further categorised into subclasses (P-IIa–e, P-IIIa–d). SVMs are structurally and evolutionarily related to physiologically and pathologically important mammalian ADAM (A Disintegrin And Metalloproteinase) proteins [9]. Together they belong to the repolysin family of the M12B clan of zinc metalloproteinases (MEROPS classification: <http://merops.sanger.ac.uk/cgi-bin/famsum?family=M12B>).

Degradation of plasma fibrinogen is one of the most pronounced clinical effects following snake bites and is caused by metallo- and serine proteinases. SVMs are directly acting fibrin(ogen)olytic enzymes, i.e., they do not require additional factors from the blood for their activity. They cleave preferentially the α - or β -chain of fibrin(ogen) and, accordingly, are referred to as α - or β -fibrin(ogen)ases [10]. SVMs are not inhibited by human serum proteinase inhibitors (serpins), which makes them very useful in the development of diagnostic tests and therapeutic agents [11]. For example, this characteristic is one of the main advantages of SVM-based anti-thrombotics over the anti-thrombotics currently used in the clinic. The latter affect coagulation indirectly by activation of the endogenous fibrin(ogen)olytic system (i.e. activating plasminogen) and are readily inhibited by serpins.

Since haemorrhage and necrosis are the most pronounced clinical effects in humans following envenomation by nose-horned viper (*Vipera ammodytes ammodytes*, Vaa) venom, the proteolytic components of this venom have been studied intensively. All four potent Vaa hemorrhagins characterised so far, Vah1, Vah3, Vah4 and ammodytagin, have pronounced fibrinogenolytic activity [12–15]. Non-hemorrhagic ammodytagin acts as a potent fibrin(ogen)ase [16], while FX activators, VAFXA-I and VAFXA-II, express low fibrinogenolytic activity [17]. A serine protease (SP) with fibrin(ogen)olytic activity, VaSP1, has recently been purified from Vaa venom [18]. We report here the purification and extensive biochemical characterisation, including complete primary structure determination, of a new α -fibrinogenolytic MP, VaF1 (V. ammodytes fibrinogenase 1). This molecule is not hemorrhagic. In standard experimental conditions it is not recognised by Vaa venom antiserum, suggesting its participation in delayed pathological effects following serotherapy of envenomed patients, possibly as an anti-coagulant.

2. Materials and methods

Crude Vaa venom was obtained from the Institute of Immunology, Inc., Croatia. All other chemicals were of analytical or sequencing grade.

2.1. Isolation of VaF1

VaF1 was isolated in the course of purification of hemorrhagic MP Vah3 as described [14]. In the last step of Vah3 purification,

FPLC on a Mono S column (binding buffer: 20 mM MES, 2 mM $CaCl_2$, 5% (w/v) glycerol, pH 6.5; linear gradient elution from 0 to 1 M NaCl in binding buffer), VaF1 was eluted at 200 mM NaCl. This fraction was tested for fibrinogenolytic and hemorrhagic activities. It was concentrated and dialysed against 20 mM Tris/HCl buffer, 2 mM $CaCl_2$, 5% (w/v) glycerol, pH 8.5 and applied onto an FPLC Mono Q column equilibrated in the same buffer. Bound proteins were eluted with a 30 min linear gradient of 1 M NaCl in the equilibration buffer at a flow rate of 1 ml/min. Proteins were monitored by absorbance at 280 nm throughout the isolation procedure.

2.2. SDS-polyacrylamide gel electrophoresis and isoelectric focussing

SDS-PAGE on 12.5%, 10% or 7.5% (w/v) polyacrylamide gels under reducing and non-reducing conditions [19] was used to analyse the isolated protein and cleavage products of different proteins hydrolysed by VaF1. Molecular mass standards used were from Fermentas (Lithuania).

IEF was performed on a Phast System (Amersham Pharmacia Biotech, Sweden) using a PhastGel isoelectric focussing (IEF) 3–9 slab-gel (0.35 × 43 × 50 mm) according to the manufacturer's instructions. pI (3.5–9.3) standards used were from the same manufacturer. Proteins were visualised by Coomassie Brilliant Blue R250 staining.

2.3. N-Deglycosylation

4 μ g of dry VaF1 were incubated with 1% (w/v) SDS at 100 °C for 5 min and mixed with 1.5% (w/v) CHAPS in 50 mM Na_2HPO_4 , pH 7.5. Half the sample was mixed with 3 U of peptide N-glycosidase F (PNGF, Roche, Germany); the other half served as control. After overnight incubation at 37 °C both samples were analysed by SDS-PAGE on 12.5% gel under reducing conditions.

2.4. Edman sequencing and mass spectrometry

N-terminal amino acid sequencing of proteins and peptides was performed on a Procise 492A Automated Sequencing System (Applied Biosystems, USA).

The molecular mass of natural VaF1 was determined by MALDI-TOF mass spectrometry (MS) as described in Ref. [15].

2 μ g of VaF1 purified by HPLC and vacuum dried was reconstituted in 10 μ l of 6 M urea in 50 mM NH_4HCO_3 . It was reduced by adding 0.25 μ l of 100 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 for 1 h at 37 °C. The mixture was then diluted with 10 μ l of 50 mM NH_4HCO_3 and alkylated with 0.8 μ l of 250 mM iodoacetamide in 50 mM NH_4HCO_3 for 45 min at room temperature in the dark. The alkylation was stopped by adding 2.5 μ l of DTT, and 50 mM NH_4HCO_3 then added to 90 μ l, followed by 10 μ l (0.05 μ g) of trypsin in 50 mM NH_4HCO_3 . Hydrolysis proceeded overnight at 37 °C. The reaction was stopped by adding formic acid to 0.1% (v/v) final concentration. The sample was desalted and concentrated using Vivapure C18 micro spin columns (Sartorius Stedim Biotech, Germany) according to the manufacturer's instructions, except that formic acid was used instead of trifluoroacetic acid. The protein hydrolysate was analysed on an ion trap mass spectrometer 1200 series HPLC-Chip-LC/MSD Trap XCT Ultra (Agilent Technologies, Germany). MS and tandem MS (MS/MS) spectra were searched against the Vaa venom gland cDNA transcripts library and the non-redundant NCBI database using the Spectrum Mill (Agilent Technologies, CA, USA) and Mascot software (Matrix Science, Ltd., UK).

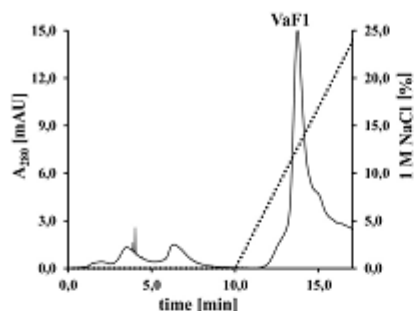


Fig. 1. Anion-exchange chromatography—the final purification step of VaF1. The FPLC Mono Q column was equilibrated in 20 mM Tris/HCl buffer, pH 8.5, containing 2 mM CaCl_2 and 5% (w/v) glycerol, and eluted with a linear gradient (0–1 M) of NaCl in the same buffer at a flow rate of 1 ml/min (dashed line).

2.5. cDNA sequence of VaF1

The cDNA sequence of VaF1 was obtained using a cDNA library of mRNA transcripts from *Vaa* venom glands and PCR primers designed according to the conserved 5' and 3' untranslated regions of mRNAs encoding P-III SVMPs, as described previously [14].

2.6. Proteolytic activity and specificity of VaF1

The fibrinolytic activity of VaF1 and the specificity of fibrinogen cleavage were examined as described [14].

The fibrinolytic activity of VaF1 was examined using the modified fibrin plate assay of [20]. Human fibrinogen (Hypen BioMed, France) was dissolved in 10 mM NaH_2PO_4 buffer, pH 7.4, containing 150 mM NaCl at a final concentration of 9.5 $\mu\text{g}/\mu\text{l}$. Fibrinogen solution, in 300 μl aliquots, was added to the wells in a 24-well plate and clotted by addition of 10 μl of α -thrombin solution (1,000 U/ml) with gentle shaking at room temperature. The plate was incubated for 3 h at 37 °C, then VaF1 (5 μg in 10 μl), or plasmin as positive control, was added on the top of fibrin clot and incubated for another 15 h. The enzymes were assayed in triplicate. Dissolution of fibrin was inspected visually.

The effect of VaF1 on bovine factor X (FX), prothrombin and plasminogen (Haematologic Technologies, Inc., USA) was tested as follows. 20 μg of each substrate was incubated with or without (control) 1 μg of VaF1 in 55 μl of 50 mM Tris/HCl, 2 mM CaCl_2 , pH 8.5 at 37 °C. 10 μl aliquots were withdrawn after 2, 4 and 24 h and analysed by 12.5% SDS-PAGE under reducing conditions. FX, α -thrombin and plasmin were used as positive controls.

The proteolytic action of VaF1 on the isolated extracellular matrix (ECM) and basement membrane (BM) proteins (ECM/BM proteins), murine laminin, human type IV collagen and bovine fibronectin (Sigma, Germany), and on soluble BM matrix (BD Matrigel Matrix Growth Factor Reduced, BD Biosciences, USA) was examined as described [14].

Hydrolysis of insulin B-chain (100 μg) by VaF1 (2 μg) in 50 mM Tris/HCl, 2 mM CaCl_2 , pH 8.5, was followed by C4-RP-HPLC analysis of reaction mixture aliquots after 0.5, 1, 3 and 24 h of incubation at 37 °C, as described in detail in Ref. [17]. Cleavage sites were determined by N-terminal sequencing of isolated fragments.

2.7. Hemorrhagic activity

Hemorrhagic activity of VaF1 (9 μg) was assayed according to [21], and as previously described [22].

2.8. Platelet aggregation assay

The effect of VaF1 on collagen-induced platelet aggregation was evaluated using the procedure of [14]. VaF1 was tested at 300 nM concentration.

2.9. Thrombin generation assay

Thrombin generation in plasma was measured fluorimetrically using Technothrombin® TGA reagents (Technoclone, GmbH, Austria). 40 μl of control plasma (TGA control high, lyophilised) were pre-incubated with 4 μl of VaF1 (1 μM final concentration) for 2 min. The coagulation cascade was then activated by addition of 10 μl of tissue factor and phospholipids (TGA reagent C (RC) Low) followed by 50 μl of a fluorogenic substrate for thrombin (TGA substrate (SUB)). Fluorescence was monitored at 37 °C at 1 min intervals for 90 min using a Victor™ X2 multilabel plate reader (Perkin Elmer, Finland). Software (www.technoclone.com) was

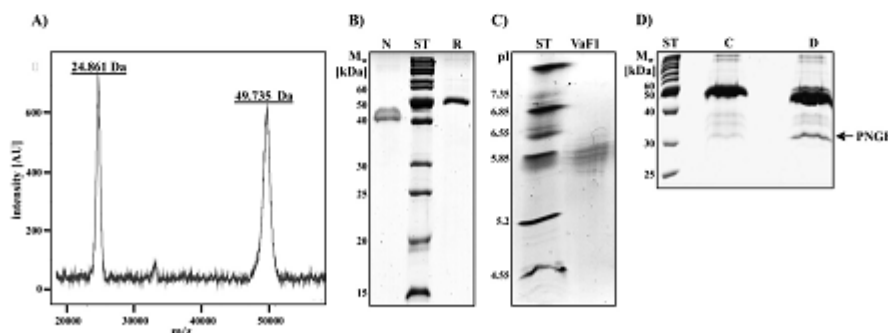


Fig. 2. Basic characterisation of VaF1. VaF1 is a monomer of 49,735 Da as determined by MALDI-TOF mass spectrometry (A) and SDS-PAGE analysis under non-reducing (N) and reducing (R) conditions (B). On non-reducing SDS-PAGE, it migrates as a slightly lighter protein. (C) VaF1 is seen, by IEF analysis, to be an acidic protein with pI values dispersed around pH 5.8. (D) VaF1 is N-glycosylated. Denatured VaF1 was treated with PNGF and analysed on a 12.5% SDS-PAGE gel under reducing conditions. The molecular mass of N-deglycosylated VaF1 (lane D) was about 3 kDa lower than that of non-treated VaF1 (lane C). ST, molecular mass standards.

1	atgttgcaagttcttttggtaactatatgcttagcagttttccatatcaaggagctct	60
-192	M L Q V L L V T I C L A V P P Y Q G S S	-173
61	ataattctggaatctgggaatgttaattgattatgaagtagtgatccacaaaaactcact	120
-172	I I L E S G N V N D Y E V V Y P Q K L T	-153
121	gcattgctcaaggagcaattcagcagcctgagcaaaagtatgaagacgcatgaat	180
-152	A L L K G A I Q Q P E Q K Y E D A M Q Y	-133
181	gaatttaagtgaaatggaaagccagtggtccttcacctagaaaaataaaggacttttt	240
-132	E F K V N G K P V V L H L E K N K G L F	-113
241	tcagaagattacagtgagactcattattccccagatggcagagaaattacacaaacct	300
-112	S E D Y S E T H Y S P D G R E I T T N P	-93
301	ccagttgaggatcactgctactatcatggacacatccagaatgatgctcacttaactgca	360
-92	P V E D H C Y Y H G H I Q N D A H L T A	-73
361	agcatcagtgcttgcaatggtttgaaaggacattccagcttcgaggggagacgtactta	420
-72	S I S A C N G L K G H F Q L R G E T Y L	-53
421	attgaacccttgaaagattcccgacagtgaaagccatgcagtcacaaatgaaaaatgta	480
-52	I E P L K I P D S E A H A V Y K Y E N V	-33
481	gaaaaagaggatgagggcccaaaaagtgtggggttaaccagactaattggaatcagat	540
-32	E K E D E G F K K C G V T Q T N W K S D	-13
541	gagcccatcaagcctctcagtttattcttactcctgaaacagcagcatatgaatgcc	600
-12	E P I K A S Q F I L T P E Q R A Y M N A	8
601	aaacaaatcattgaagcttgcaatagttgtggacacgtaatgttcaggaaatcacccggg	660
9	N K Y I K L A I V V D N V M F R K Y T G	28
661	aatttcactgctataagaacagaatataatgaattgtcaacttttaactctgatttac	720
29	N F T A I R T R I Y E I V N T L N L I Y	48
721	acaattttgaatattcatatagcactggttttccatagaatttgggtccaaaggagattcg	780
49	T I L N I H I A L V F L E I W S K G D S	68
781	attaacgttcaatcagtagtggtggttactttgaaactcatttgagagatggagagaga	840
69	I N V Q S V V D V T L N S F G E W R E R	88
841	gatttgctgaatcgaaaaagacatgataatgctcagttactcacgggcatttaactcaat	900
89	D L L N R K R H D N A Q L L T G I N F N	108
901	ggagacactataggattgtgtttgtgggagcagtgatgccggaagaatctgttagga	960
109	G D T I G F G F V G S M C I P K K S V G	128
961	attgttcaggatcatagtaaaacacatcttttgggttgcaactacaatggcccatgagctg	1020
129	I V Q D H S K T H L L V A T T M A H E L	148
1021	ggtcataatctgggcattaatcatgagtgagattcctgtacttgccaggcctaactgc	1080
149	G H N L G I N H D G D S C T C Q A N S C	168
1081	attatggctgcgaactaagccatcaaccttcctatcagttcagcgattgtgatatataat	1140
169	I M A A K L S H Q P S Y Q F S D C S I N	188
1141	gaactttggatgtatcttattagtcataccccaagatgcattctcaatgaaccttgaag	1200
189	E L W M Y L I S H T P R C I L N E P L T	208
1201	acagatgtgtttcacctgcagttgttgaaattacgttggtggaggaaggagaagaatgt	1260
209	T D V V S P A V C G N Y V V E E G E E C	228
1261	gactgtggtcttttatggtattgtcgaaatccgtgtgtgtgctgcaacctgtaaactg	1320
229	D C G S L W Y C R N P C C D A A T C K E	248
1321	aaaccaggggcagagtggtggagtggtgtgtgtgtttacaaatgcagatttgtgacagca	1380
249	K P G A E C G D G V C C Y Q C R F V T A	268
1381	ggaacagtgatgcccggccagcaagaagtgagtggtgacatccctgaatactgcagtgccaa	1440
269	G T V C R P A R S E C D I P E Y C S G Q	288
1441	tctgttgagtggtccatggatcatatccagaagaatggaaaacctgacctaatgaaccac	1500
289	S V E C P M D H I Q K N G K P C L M N H	308
1501	ggttattgctacaatggagatgccccatcatgataaccaatgtattgtctgtgtggggg	1560
309	G Y C Y N G R C P I M I H Q C I A L W G	328
1561	ccaggtacaactgtgtcttcagatgtatgttttcagagaaaacgaaagcgggcaaggttat	1620
329	P G T T V S S D V C F Q R N E S G Q G Y	348
1621	tcctactgcagaagggaaaataatcaaaacattccatgtgcaccacagagtgtaaatgt	1680
349	S Y C R R E N N Q N I P C A P Q D V K C	368
1681	ggaaggttatactgcaaatctcataatgtcaacacacttccatgcaatttcaaatattca	1740
369	G R L Y C K F H N V N T L P C N F K Y S	388
1741	gattttgccccgattatggattgtgtgtgacatggaacaaatgtggagatggaaggtgc	1800
389	D F A P D Y G L V D H G T K C G D G K V	408
1801	tgcaacagtaaacaggcagtggtgtgtgtgaatacagcctactaa	1845
409	C N S N R Q C V D V N T A Y stop	422

used to determine peak thrombin generation in nmol/l. The area under the curve represented endogenous thrombin potential.

2.10. Western blot analysis

0.7 µg/lane of VaF1 and 2 µg/lane of whole *Vaa* venom were separated by SDS-PAGE on 12.5% (m/v) polyacrylamide gels under reducing conditions, at 20 mA per gel for 90 min. Proteins were transferred to a nitrocellulose membrane (NC2, Serva, Germany) at room temperature at 200 mA per gel for 90 min in Towbin transfer buffer (4 mM Tris/HCl, pH 7.5, 12 mM NaCl and 20% (v/v) methanol). Following transfer, the membrane was incubated overnight with either rabbit anti-*Vaa* venom or anti-H serum [23,24] at a dilution of 1:1000.000 in 0.5% (w/v) Western blocking solution (WBS, Roche, Germany) at 4 °C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgGs (1:10.000 in 0.5% (w/v) WBS) for 45 min at room temperature. The bands were visualised with the ECL Lumi-LightPLUS Western Blotting substrate (Roche, Germany) and exposure to Amersham Hyperfilm ECL (GE Healthcare, UK) following the manufacturer's instructions.

3. Results

3.1. Purification and basic characterisation of VaF1

A high molecular mass fibrinolytic MP was isolated from the venom of *Vaa* and named *V. ammodytes* fibrinogenase 1 (VaF1) using the well established procedures for purification of proteins from *Vaa* crude venom [12,17]. VaF1 was eluted from hydroxyapatite chromatography together with fibrinolytic and hemorrhagic MP VaH3. The two SVMPs were separated by FPLC on a Mono S column at pH 6.5 [14]. Minor impurities present in the VaF1 fraction were removed by FPLC on a Mono Q column at pH 8.5 (Fig. 1). The homogeneity of the isolated protein and its molecular mass were determined by SDS-PAGE and MALDI-TOF MS analysis (Fig. 2A). VaF1 migrated on a non-reducing SDS-PAGE gel as a protein of about 45 kDa. Under reducing conditions the mass of VaF1 was only slightly higher, revealing its monomeric nature (Fig. 2B). Its molecular mass determined by mass spectrometry is 49,735 Da. It is an acidic protein with multiple isoelectric points scattered around pH 5.8 (Fig. 2C). The diffuse isoelectric point is due to glycosylation – its apparent molecular mass was reduced by about 3 kDa when treated with PNGF (Fig. 2D). 170 µg of pure VaF1 was isolated from 1 g of crude *Vaa* venom.

3.2. Primary structure of VaF1

Edman sequencing of the natural VaF1 failed, leading to the assumption that the N-terminal amino acid residue of VaF1 is blocked. The reduced and alkylated protein was then hydrolysed with trypsin and the resulting peptides sequenced by MS/MS analysis. The sequences obtained were identified as parts of a transcript encoding a P-III MP PCR-amplified from a *Vaa* venom gland cDNA library. The identified VaF1 peptides cover 34% of the mature part of this amino acid sequence-translated cDNA transcript. They correspond to fragments of the MP, dis-like and Cys-rich domains of the P-III SVMP molecule (Fig. 3). Being a

monomeric protein, as demonstrated by SDS-PAGE analysis (Fig. 2), it belongs to the P-IIIa subclass of the P-III SVMPs.

The complete nucleotide sequence of the VaF1 precursor is composed of 1845 base pairs (Fig. 3), coding for a precursor protein of 614 amino acids. Its prepro-segment consists of two highly conserved parts, a 20-amino acid residue signal peptide and a 172-amino acid residue pro-peptide. The latter includes the Cys-switch motif PKKCGVT. Judged by its similarity to closely related SVMPs, the mature protein starts at Glu and is 422 residues long. The first residue of the mature protein, Glu1, is most probably, as in the closely related SVMPs, post-translationally modified to pyroglutamate [25]. VaF1 is an N-glycosylated protein and two potential N-glycosylation sites are found in its sequence.

The non-redundant NCBI database was searched with the full-length precursor and with the mature form of VaF1. As expected, it was found to show the highest identity with some P-III SVMPs (Fig. 4). Comparing only mature forms of these proteins, VaF1 is most similar to the three tentative P-III MPs from *Echis coloratus* (AD147652.1, AD147630.1, AD147641.1), showing 82% amino acid identity. 79% of the amino acid residues are identical to the two predicted P-III MPs from *Echis carinatus* *sochureki* (AD147588.1, AD147601.1). VLAIP-A (Q4VM08.1) and VLAIP-B (Q4VM07.1), subunits of the heterodimeric P-III MP from the venom of *Macrovipera lebetina*, and VaH3 (KC007435), the homodimeric P-IIIc MP from the same venom as VaF1, are less similar, with 62–65% identity.

3.3. Proteolytic specificity of VaF1

After incubating insulin B-chain with VaF1 for more than 24 h, cleavage of only two peptide bonds was detected, Ala14–Leu15 and Tyr16–Leu17. They were hydrolysed after only 30 min of incubation.

3.4. Fibrinogenolytic activity

VaF1 hydrolyses fibrinogen (Fig. 5A). The band corresponding to the A α -chain of fibrinogen disappeared in about 30 min of incubation with VaF1, while the B β - and γ -chains remained intact even after 3 h. The N-terminal sequence of the 15 kDa A α -chain degradation product constitutes the N-terminus of VaF1, while the 13 kDa fragment results from cleavage of the Lys413–Leu414 bond. As expected, pre-incubation of the enzyme with EDTA blocked the fibrinolytic activity of VaF1, whereas pefabloc, an inhibitor of SPs, had no effect on VaF1 proteolytic activity (not shown).

In the fibrin plate assay no fibrin degradation was observed with 5 µg of VaF1. For comparison, in a control experiment in which plasmin was used instead of VaF1, fibrin was completely dissolved, i.e., degraded.

3.5. Proteolytic actions of VaF1 on FX, prothrombin and plasminogen

The two major coagulation factors, FX and prothrombin, and the fibrinolysis factor, plasminogen, were only partially hydrolysed by VaF1 *in vitro* (Fig. 5B–D). FX and plasminogen were degraded also very slowly. None of the degradation products detected by SDS-PAGE analysis had the same apparent molecular mass as the heavy chains of the activated factors, FXa, α -thrombin and plasmin.

Fig. 3. Nucleotide and deduced protein sequences of VaF1 precursor. VaF1 precursor starts with a 20 amino acid residue signal peptide (grey letters), followed by a pro-sequence (residues 172 to –1), then the mature part (residues 1–422). The maturation-controlling sequence, the so-called Cys-switch motif, is underlined. A Glu residue, the predicted starting amino acid residue of the VaF1 mature form, is highlighted black. This residue is probably post-translationally modified to pyroglutamate. VaF1 was partially sequenced on the protein level using ESI ion trap MS. These parts are grey highlighted and constitute 34% of the mature VaF1 sequence. The consensus N-glycosylation sequences are boxed. The accession number of its cDNA and the corresponding amino acid sequence of VaF1 in the GenBank database is KM886926.

VaF1	1	DQRYVLSKPKYIKLVIAVYDQVGRSLIT	TRTRIVSVNTLN	TVVNTN	HALV	STIM	SDS	INVC	VDVPTL	SFGEMRRDL	90
MP-Ec	1	DQRYVLSKPKYIKLVIAVYDQVGRSLIT	TRTRIVSVNTLN	TVVNTN	HALV	STIM	SDS	INVC	VDVPTL	SFGEMRRDL	90
MP-Ecs	1	DQRYVLSKPKYIKLVIAVYDQVGRSLIT	TRTRIVSVNTLN	TVVNTN	HALV	STIM	SDS	INVC	VDVPTL	SFGEMRRDL	90
VLAIP-B	1	DQRYVLSKPKYIKLVIAVYDQVGRSLIT	TRTRIVSVNTLN	TVVNTN	HALV	STIM	SDS	INVC	VDVPTL	SFGEMRRDL	90
VLAIP-A	1	DQRYVLSKPKYIKLVIAVYDQVGRSLIT	TRTRIVSVNTLN	TVVNTN	HALV	STIM	SDS	INVC	VDVPTL	SFGEMRRDL	90
VaH3	1	DQRYVLSKPKYIKLVIAVYDQVGRSLIT	TRTRIVSVNTLN	TVVNTN	HALV	STIM	SDS	INVC	VDVPTL	SFGEMRRDL	90
VaF1	91	LNKPHDNAQLLTGINFNP	PTL	PTL	PTL	PTL	PTL	PTL	PTL	PTL	180
MP-Ec	91	LNKPHDNAQLLTGINFNP	PTL	PTL	PTL	PTL	PTL	PTL	PTL	PTL	180
MP-Ecs	91	LNKPHDNAQLLTGINFNP	PTL	PTL	PTL	PTL	PTL	PTL	PTL	PTL	180
VLAIP-B	91	LNKPHDNAQLLTGINFNP	PTL	PTL	PTL	PTL	PTL	PTL	PTL	PTL	180
VLAIP-A	91	LNKPHDNAQLLTGINFNP	PTL	PTL	PTL	PTL	PTL	PTL	PTL	PTL	180
VaH3	91	LNKPHDNAQLLTGINFNP	PTL	PTL	PTL	PTL	PTL	PTL	PTL	PTL	180
VaF1	181	QPSDCSIN	IN	IN	IN	IN	IN	IN	IN	IN	270
MP-Ec	181	QPSDCSIN	IN	IN	IN	IN	IN	IN	IN	IN	270
MP-Ecs	181	QPSDCSIN	IN	IN	IN	IN	IN	IN	IN	IN	270
VLAIP-B	181	QPSDCSIN	IN	IN	IN	IN	IN	IN	IN	IN	270
VLAIP-A	181	QPSDCSIN	IN	IN	IN	IN	IN	IN	IN	IN	270
VaH3	181	QPSDCSIN	IN	IN	IN	IN	IN	IN	IN	IN	270
VaF1	271	YCRPANGCCV	SD	CT	CT	CT	CT	CT	CT	CT	360
MP-Ec	271	YCRPANGCCV	SD	CT	CT	CT	CT	CT	CT	CT	360
MP-Ecs	271	YCRPANGCCV	SD	CT	CT	CT	CT	CT	CT	CT	360
VLAIP-B	271	YCRPANGCCV	SD	CT	CT	CT	CT	CT	CT	CT	360
VLAIP-A	271	YCRPANGCCV	SD	CT	CT	CT	CT	CT	CT	CT	360
VaH3	271	YCRPANGCCV	SD	CT	CT	CT	CT	CT	CT	CT	360
VaF1	361	CAQDVVKGRILY	CF	CF	CF	CF	CF	CF	CF	CF	422
MP-Ec	361	CAQDVVKGRILY	CF	CF	CF	CF	CF	CF	CF	CF	422
MP-Ecs	361	CAQDVVKGRILY	CF	CF	CF	CF	CF	CF	CF	CF	422
VLAIP-B	361	CAQDVVKGRILY	CF	CF	CF	CF	CF	CF	CF	CF	422
VLAIP-A	361	CAQDVVKGRILY	CF	CF	CF	CF	CF	CF	CF	CF	422
VaH3	361	CAQDVVKGRILY	CF	CF	CF	CF	CF	CF	CF	CF	422

Fig. 4. The sequence of the mature form of VaF1 aligned with sequences of the most similar SVMPs. These are: MP-Ec (AD47652.1) from *Echis carolinus*, MP-Ecs (AD47588.1) from *Echis carolinus* suchareki, VLAIP-A (Q4VM08.1) and VLAIP-B (Q4VM07.1) from *Macrovipera lebetina* venom, and VaH3 from *Vipera* venom (KC007435). In the alignment, position-dominant amino acid residues are highlighted black and position-similar residues highlighted grey. The percentage of sequence identity between VaF1 and a compared SVMP is indicated at the end of each row.

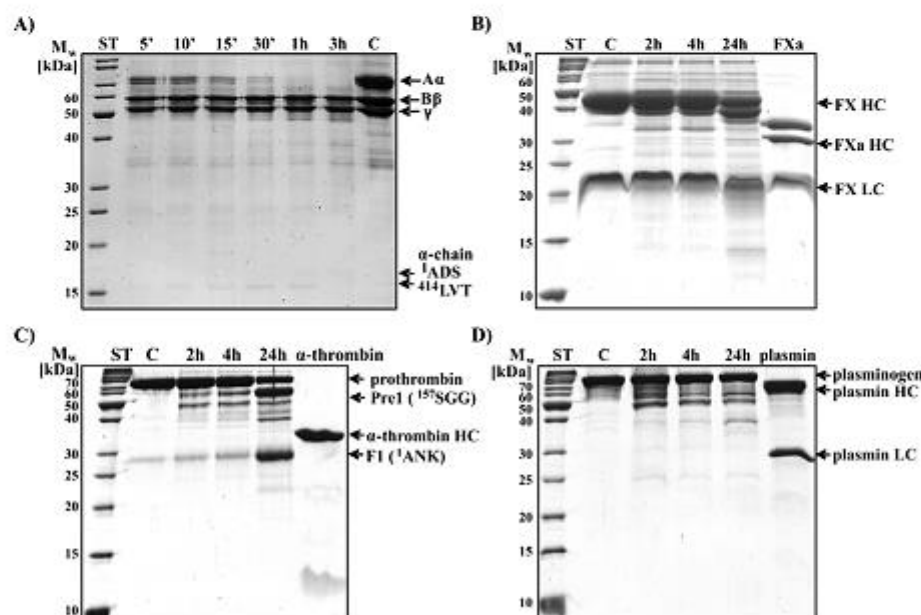


Fig. 5. Proteolytic activity of VaF1 against components of the blood coagulation and fibrinolytic systems. Fibrinogen (panel A), FX (panel B), prothrombin (panel C) and plasminogen (panel D) were incubated with VaF1 at 37 °C for the times indicated, and then analysed by SDS-PAGE under reducing conditions. Experimental details are described in Section 2.6. Active forms of the coagulation factors, FXa, α-thrombin and plasmin, were electrophoresed as references to show that VaF1 cleaved these factors in a non-activating manner. In control experiments (C), substrates were incubated 24 h without VaF1. ST, molecular mass standards. F1 designates the activation fragment 1 of prothrombin.

Thus, under the experimental conditions used, VaF1 cleaved these factors but did not activate them. The heavy chain of FX was more susceptible to hydrolysis by VaF1 than the light chain whose hydrolysis was observed only after prolonged incubation. Prothrombin was also not a good substrate for VaF1. Two major cleavage products, of 60 and 28 kDa, accumulated after extended incubation with VaF1. N-terminal sequencing showed them to be prothrombin-1 and fragment 1, indicating the cleavage of prothrombin by VaF1 at the Ser157–Gly158 bond. Incubation of plasminogen with VaF1 resulted in very limited proteolysis, giving rise to multiple products with molecular masses between 40 and 60 kDa. The cleavage pattern changed little with time of reaction.

3.6. Proteolysis of ECM and BM proteins

The hemorrhagic action of SVMPs depends on their ability to hydrolyse proteins of BM and ECM. Although devoid of hemorrhagic activity, VaF1 was tested *in vitro* for its effect on isolated major protein constituents of ECM and BM, laminin, collagen IV and fibronectin. Its effect was also determined on Matrigel, a solubilised preparation of BM that better simulates the actual structural complexity of these substrates *in vivo* where the individual components are intertwined in a complex network that limits their accessibility to SVMPs. After 3 h incubation with VaF1 at 37 °C, collagen IV and fibronectin were partially hydrolysed (Fig. 6A). However, laminin, either isolated or as a constituent of Matrigel, resisted hydrolysis by VaF1, even after 24 h incubation (Fig. 6B and C). However, in the experiment with Matrigel, cleavage by VaF1 of nidogen, another ECM/BM protein, was observed. Edman degradation of the major degradation product showed that it was hydrolysed at the Ser322–Phe323 bond (Fig. 6C).

3.7. Pharmacological activity of VaF1

VaF1 displayed no hemorrhagic activity when tested in rats. Intradermal injection of 9 µg of the enzyme induced no pathological changes.

Pre-incubation of platelet-rich plasma with 300 nM VaF1 did not inhibit collagen-dependent aggregation of platelets. 1 µM VaF1 also had no effect on blood coagulation in a thrombin generation assay.

3.8. Western blot analysis of VaF1

Serum from rabbits immunised with a hemorrhagic fraction of Vaa venom (anti-H serum) recognised purified VaF1 in Western blot analysis but failed to detect it in the whole venom. When

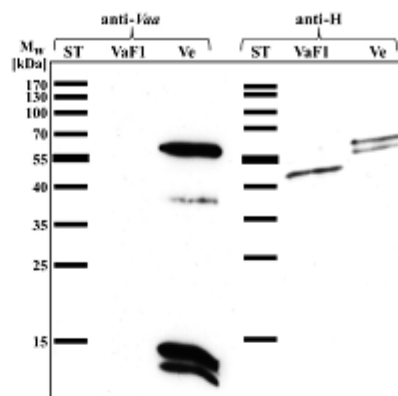


Fig. 7. Western blot analysis of VaF1. Antisera against whole Vaa venom (anti-Vaa) and against its isolated hemorrhagic fraction (anti-H) were produced in rabbits. SDS-PAGE analysis of VaF1 (VaF1, 0.7 µg/lane) and of whole Vaa venom (Vv, 2 µg/lane) was performed under reducing conditions. Following transfer of proteins from gel onto nitrocellulose membrane, immunodetection was performed by incubating the membrane, first with rabbit anti-H or anti-Vaa serum, and then with HRP-conjugated secondary anti-rabbit IgGs. Antibody-recognised bands were detected on a photographic film using the ECL Lumi-LightPLUS system. Anti-H, but not anti-Vaa serum, recognised VaF1, however not in the whole venom and only when analysed as a pure protein. ST, molecular mass standards.

serum from a rabbit immunised with the whole Vaa venom (anti-Vaa venom serum) was used instead, even pure VaF1 was not immunodetected (Fig. 7). This shows that, in spite of the relatively high level of identity between VaF1 and some other venom SVMPs (e.g. VaH3 that is 62% identical to VaF1), these proteins are at most weakly immunologically cross-reactive. We can also conclude that the low abundance of this molecule in the venom—and maybe its low immunogenicity—are the reasons for the negligible production of anti-VaF1 antibodies following immunisation with whole venom. VaF1 was, however, enriched in the hemorrhagic (H) fraction of the venom and, when immunisation was performed with this fraction, specific antibodies were formed.

4. Discussion

Previously, only one potent fibrinolytic and non-hemorrhagic P-III MP from the Vaa venom, ammodysase, had

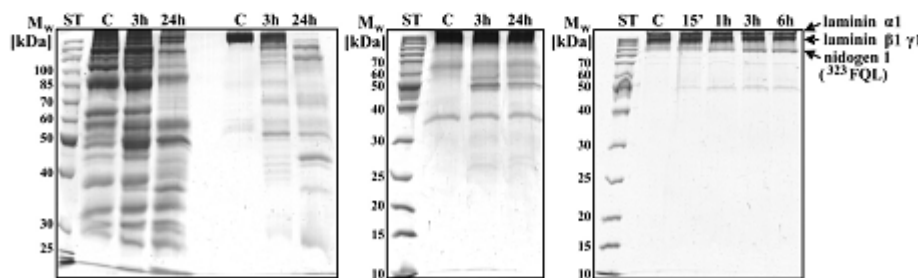


Fig. 6. Hydrolytic activity of VaF1 on ECM/BM proteins. Isolated components of ECM/BM, collagen IV, fibronectin and laminin, were incubated with VaF1 in a mass ratio 25:1 at 37 °C for the times indicated, and the reaction products analysed by SDS-PAGE under reducing conditions. VaF1 hydrolysed collagen IV (panel A, left) and fibronectin (panel A, right) but not laminin (panel B). Matrigel and VaF1 were incubated in a mass ratio of 40:1. SDS-PAGE analysis (panel C) revealed one major cleavage product, identified by Edman sequencing as a fragment of nidogen starting at Phe323. In this case also, laminin remained untouched. In control experiments (C), substrates were incubated for 24 h without VaF1. ST, molecular mass standards.

been isolated and characterised [16]. A second fibrinogenolytic and non-hemorrhagic P-III MP from the venom of *Vaa*, termed VaF1, is here described. The 1845 base pairs of the cDNA coding for the complete VaF1 precursor encode a prepro-protein of 614 residues (Fig. 3). The sequence starts with a 20 residue export signal peptide, characteristic of extracellular proteins [26]. This is followed by a 594 residue zymogen sequence. Given the structural similarity to other SVMPs and matrix MPs, the proteolytic activity of VaF1 zymogen in the venom gland appears to be prevented by a Cys-switch mechanism, common to these protein molecules [6]. VaF1 zymogen thus harbours a modified Cys-switch motif (whose canonical form is the PKMCGV sequence) in its pro-peptide region. By the Cys-switch mechanism, the Cys residue in this motif interacts with the Zn^{2+} ion in the active site of the proteinase, resulting in its inhibition. The Met in this sequence is clearly not essential for the inhibition of zymogen enzymatic activity, since it is replaced by a Lys in VaF1, and by a Thr in VaH3, without noticeable impact on the processing of these precursor molecules [14]. The mature form of VaF1 is 422 amino acid residues long and composed of three domains typical of P-III MPs, an MP, a dis-like and a Cys-rich domain. VaF1 has been shown to be a monomeric protein of 49.7 kDa (Fig. 2), qualifying it as a P-IIIa subclass MP, the same as ammodytase. Six conserved Cys residues are present in its MP domain (Fig. 4) and, presumably, follow the pairing style typical of P-III SVMPs [7]. Two characteristic active site sequences in MPs are also present in VaF1—the Zn^{2+} -chelating sequence, HELGHNLGINHD, and the so-called Met-turn, CMAA. These structural elements classify VaF1 to the metzincin subfamily of the repolysin family of zinc MPs.

The molecular mass of the mature VaF1 determined using PNGF is 47.1 kDa. The discrepancy between this and the MS-determined mass is 2.6 kDa and stems from the N-glycosylation (Fig. 2). From the sequence of VaF1, carbohydrates can attach to the molecule on two sites, Asn29 in the MP domain and/or Asn342 in the Cys-rich domain (Figs. 3 and 4). The extent of glycosylation of VaF1 is much lower than that of ammodytase, in which the mass of the carbohydrate moiety is about 9 kDa (A. Leonardi, personal communication).

VaF1 shares a high degree of sequence identity with repolysins. The highest identity found was 82% with three SVMPs from *E. coloratus* with as yet unknown functions [27]. VaF1 shows about 60% identity to the two subunits of VLAIP, a heterodimeric apoptosis-inducing SVMP from *M. lebetina* [28], and to hemorrhagic VaH3 from *Vaa* that exists as a homodimer [14].

Fibrinogen is one of the major proteins in the blood. It is composed of two identical modules (each consisting of α -, β - and γ -chains, linked together by disulphide bonds) facing each other at the N-terminal ends [29]. On proteolytic activation by α -thrombin it polymerises into a highly organised fibrin network that forms the insoluble blood clot that arrests the bleeding [30]. A number of high molecular mass SVMPs with fibrinogenolytic activity have been isolated from different snake venoms, however, only a few are devoid of hemorrhagic activity, one of them being VaF1. The group of P-IIIa MPs comprises ammodytase from *Vaa* [16], AAV1 from *Deinagkistrodon acutus* [31], atrase B from *Naja atra* [32] and NN-PF3 from *Naja naja* [33]. Among P-IIIc MPs these are characteristics of VLAIP from *M. lebetina* [28], TSV-DM50 from *Trimeresurus stejnegeri* [34] and AHMP from *Gloydus halys* venom [35]. In contrast to ammodytase, NN-PF3 and AHPM, VaF1 does not degrade fibrin clots. VaF1 selectively cleaves the α -chain of fibrinogen, so it is exclusively an α -fibrinogenase, like atrase B and NN-PF3 [32,33]. Cleavage positions in fibrinogen have been determined only for VaF1, ammodytase [16] and VLAIP [28]. Fibrinogen α -chains are almost twice as long as β - and γ -chains, and their C-terminal domains (α C-domains), which protrude from the

fibrinogen molecule, are more susceptible to proteolysis by SVMPs. In this region, VaF1 and VLAIP were found to hydrolyse the same Lys413–Leu414 peptide bond. It appears that this site is frequently attacked by SVMPs regardless of their hemorrhagic potential or the structural class to which they belong [13–15,36–38]. Ammodytase cleaves fibrinogen in the same region as the SVMPs described above but with diverse peptide bond specificities [16]. Following the observation that fibrinogen, nicked in its α C-domain by SVMPs, forms a loose fibrin clot that is more prone to lysis [39], it was found that fibrinogen α C-domains have a very important function in lateral aggregation and crosslinking of protofibrils in the process of fibrin formation [40]. Moreover, the α C-domain mediates interactions with some other plasma proteins. This domain harbours, for example, binding sites for fibronectin, FXIII, plasminogen and tissue plasminogen activator. By binding to the α C-domains of fibrin, plasma proteins are incorporated into a fibrin clot. The α C-domains of fibrinogen and fibrin also bind receptors (integrins) on cells. In this way, the former play an important role in the processes of fibrinolysis and wound healing. VaF1, by its specific proteolysis of fibrinogen, could prevent the formation of physiologically effective fibrin clots, normal fibrinolysis and wound healing. Its anti-coagulant potential is further emphasised by its action on other coagulation factors—VaF1 partially hydrolyses FX, prothrombin and plasminogen—however, not to their respective active forms (Fig. 5). For example, like hemorrhagic MPs from *Vaa* venom, VaF1 cleaved the activation peptide 1 from prothrombin (FII). The resulting prothrombin-1 cannot bind to phospholipid membranes to form the prothrombinase complex together with FXa and FVa, so that α -thrombin (FIIa), crucially important for fibrin polymerisation, activation of platelets and several blood coagulation factors, is not formed.

P-III class SVMPs affect platelet aggregation by different mechanisms [4]. SVMPs were found to bind to and/or cleave platelet adhesion receptors, glycoprotein Ib (GPIb), GPIIb/IIIa, integrin $\alpha_2\beta_1$ and integrin $\alpha_5\beta_3$ or their ligands, von Willebrand factor, collagen or fibrinogen. Like ammodytase, VaF1 is not able to inhibit collagen-induced aggregation of platelets. Although it possesses a SECD sequence in its dis-like domain, which has been indicated as the sequence responsible for binding to collagen and consequently preventing collagen-induced aggregation of platelets [41], this is obviously not functional in VaF1 probably due to a steric reason. Although information about the structures and functions of SVMPs is accumulating rapidly, no distinct structural motif has been revealed that would clearly distinguish between a hemorrhagic and a non-hemorrhagic P-III SVMP. In general, the P-III hemorrhagic SVMPs are more potent in their pathologic action than the P-I hemorrhagic SVMPs. It was suggested that this is due to the more complex action of the former, involving, besides the catalytic action, the assistance of the non-catalytic domains [42]. Several lines of evidence support the hypothesis that SVMPs cause haemorrhage by weakening micro-vessel walls by hydrolysis of the BM and its surrounding ECM proteins [43]. Haemodynamic forces, imposed by blood streaming through the vessel, ultimately disrupt the damaged vessels and blood extravasates. Although devoid of hemorrhagic activity under the experimental conditions used, VaF1 is, like ammodytase, able to cleave some proteins of the BM and ECM *in vitro* (Fig. 6). It cleaved collagen IV, nidogen and fibronectin, however, much more slowly and less effectively than hemorrhagic SVMPs from *Vaa* venom [13–15]. In nidogen the preferred cleavage site of VaF1 is at Ser322–Phe323. This bond is also preferentially attacked by hemorrhagic *Vaa* SVMPs. However, the latter enzymes also cleave nidogen at positions Tyr352–Asn353 and Val422–Phe423, which is essential for destabilisation of the BM structure [44,45]. The inability of VaF1 to induce haemorrhage *in vivo* is thus due to the low degree of hydrolysis of BM and ECM, probably the

consequence of a too narrow proteolytic specificity and/or inability to recognise substrate exosites by its dis-like and Cys-rich domains.

VaF1 in the venom induced only marginal (if any) production of specific anti-VaF1 antibodies on immunisation. The fact that anti-H serum, which recognised purified VaF1, also failed to detect this molecule in the whole venom (Fig. 7) implies that the reason is in an amount of this molecule in the venom that is insufficient to induce an effective immune response. In addition, VaF1 showed at most weak immunological cross-reactivity with other SVMs in the venom (Fig. 7), although some of them are highly similar to VaF1 (e.g., VaH3 and VaF1 have 62% of amino acid residues identical). Venom components that are not recognised by specific anti-venoms can escape anti-venom serotherapy of patients and cause past-treatment complications. VaF1 is a good candidate for such a component.

5. Conclusions

A non-hemorrhagic proteinase, VaF1, has been purified from the venom of *Vaa*, the most poisonous snake in Europe. It belongs to the P-IIIa subclass of SVMs, being a monomeric protein composed of three distinct domains—an MP, a dis-like and a Cys-rich domain. The complete amino acid sequence of VaF1 precursor was deduced by amplifying and sequencing its cDNA. We showed that VaF1 only weakly, and only at a limited number of sites, degrades some of the BM and ECM components, which is the main reason that it is not hemorrhagic. However, it hydrolyses some plasma proteins involved in blood coagulation—fibrinogen, FX, prothrombin and plasminogen—which demonstrates its anti-coagulant potential *in vivo*. Despite possessing the collagen-binding sequence in the dis-like domain, VaF1 was not able to interfere with collagen-induced aggregation of platelets. Due to its low level in the venom, and possibly also low immunogenicity, VaF1 elicited a negligible immune response on immunisation of animals with *Vaa* venom. Moreover, VaF1 cross-reacts immunologically at most weakly with similar SVMs in the venom. As such it can cause post-serotherapy complications in patients, for example anti-coagulant action. These, however, can be avoided by supplementing *Vaa* anti-venom with specific anti-VaF1 antibodies raised against the purified antigen.

Conflict of interest

The authors have no conflicts of interest to declare.

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3.5 Znanstvena publikacija 5: Prvi inhibitor intrinzičnega tenaznega kompleksa s strukturo serinske proteaze ponuja nov pristop v antikoagulantni terapiji

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Anomalije v hemostazi lahko vodijo do prekomernega strjevanja krvi, kar lahko privede do tvorbe krvnega strdka, najpogosteje v venah. Nastali strdek lahko prepreči pretok krvi skozi veno ali pa je vzrok za pljučno embolijo. Skupno ime za kardiovaskularne težave s takim ozadjem je venska tromboembolija. Venska tromboza je posledica pretiranega strjevanja krvi, ki je gensko pogojena ali pa posledica okoljskih dejavnikov.

Za zdravljenje venske tromboembolije se uporabljajo antikoagulantna zdravila, kot so heparin in antagonisti vitamin K ali tarčno usmerjena zdravila, ki specifično inhibirajo delovanje enega koagulacijskega faktorja, na primer trombina (dabigatran) ali FXa (apixaban ali rivaroxaban). Žal nobeno od omenjenih zdravil ni popolnoma varno zaradi nezaželenih stranskih učinkov, kot so resne krvavitve, interakcij s hrano in pomanjkanja antidotov v slučaju predoziranja. Boljši, tj. bolj varni, antitrombotiki se zato intenzivno iščejo. Kot zelo zanimiv kandidat se je izkazal encimsko neaktiven protein iz modrasovega strupa z izrazitim antikoagulantnim delovanjem. Ker je molekula izolirana iz strupa *Vaa* in je strukturni homolog SP, smo jo poimenovali *VaaSPH-1*. V tem članku smo raziskali biokemijske značilnosti *VaaSPH-1* in razkrili mehanizem njegove antikoagulantne aktivnosti. *VaaSPH-1* je glikoprotein z navidezno molekulsko maso 35 kDa in heterogeno pl v območju pH 6–9,5. Eksperimentalno smo določili 36 % celotnega aminokislinskega zaporedja *VaaSPH-1*, celotno strukturo pa smo določili iz ustreznega nukleotidnega zaporedja. Sekvenca je razkrila, da od treh aminokislin, ki tvorijo klasično katalitično triado pri SP, Ser-His-Asp, pri *VaaSPH-1* manjkata prvi dve.

Ugotovili smo, da se *VaaSPH-1* veže na negativno nabite fosfolipide, vendar smo pokazali, da njegova antikoagulantnost ni posledica te lastnosti. Pokazali smo, da se *VaaSPH-1* veže na več koagulacijskih faktorjev (protrombin, FVII in FVII(a), FVIIIa, FIX in FIX(a) ter FX in FXa) in tako inhibira delovanje protrombinaznega in obeh tenaznih kompleksov, intrinzičnega in ekstrinzičnega. *VaaSPH-1* je bistveno bolj podaljšal aktivirani parcialni tromboplastinski čas kot protrombinski čas, kar je kazalo, da je njegova primarna tarča v koagulacijski kaskadi koagulacijski faktor notranje koagulacijski poti. Ugotovili smo, da je ta tarča FVIIIa. Vezava *VaaSPH-1* FVIIIa onemogoči vezavo FIXa na FVIIIa in s tem tvorbo intrinzičnega tenaznega kompleksa, ki je odgovoren za aktivacijo FX. Rezultat je inhibicija strjevanja krvi. Antikoagulant, ki deluje na notranjo pot strjevanja krvi, so bolj varni za uporabo v medicini in *VaaSPH-1* s tega vidika pomeni idealno izhodišče za njihov razvoj.

Razvoja nove vrste varnih antikoagulantov za medicinsko uporabo smo se lotili s pomočjo molekularnega modeliranja. Najprej smo izdelali prostorski (3D) model molekule *VaaSPH-1* in nato še model intrinzičnega tenaznega kompleksa, v katerem smo FIXa nadomestili z *VaaSPH-1*. S pomočjo modela kompleksa smo lahko natančno določili stično površino med *VaaSPH-1* in FVIIIa, kar je osnova za načrtovanje malih molekul z antikoagulantno aktivnostjo za uporabo v medicini.

Moj prispevek k znanstveni publikaciji je bilo celotno eksperimentalno delo, razen sekvenčnih analiz in molekularnega modeliranja. Prispevala sem tudi pri pisanju članka, s pripravo prvega osnutka in pri pripravi slik.

The First Intrinsic Tenase Complex Inhibitor with Serine Protease Structure Offers a New Perspective in Anticoagulant Therapy

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Abstract

Components of the intrinsic blood coagulation pathway, among them factor VIIIa (FVIIIa), have been recognized as suitable therapeutic targets to treat venous thromboembolism, pathological process behind two very serious cardiovascular diseases, deep vein thrombosis and pulmonary embolism. Here, we describe a unique glycoprotein from the nose-horned viper (*Vipera ammodytes ammodytes* [Vaa]) venom, Vaa serine proteinase homolog 1 (VaaSPH-1), structurally a serine protease but without an enzymatic activity and expressing potent anticoagulant action in human blood. We demonstrated that one of its targets in the blood coagulation system is FVIIIa of the intrinsic tenase complex, where it antagonizes the binding of FIXa. Anticoagulants with such characteristics are intensively sought, as they would be much safer for medical application as the contemporary drugs, which frequently induce excessive bleeding and other complications. VaaSPH-1 is unlikely to be orally available for chronic usage as it has molecular mass of 35 kDa. However, it represents a very promising template to design low molecular mass FVIIIa-directed anticoagulant substances, based on structural features of the interaction surface between VaaSPH-1 and FVIIIa. To this end, we constructed a three-dimensional model of VaaSPH-1 bound to FVIIIa. The model exposes the 157-loop and the preceding α -helix as the most appropriate structural elements of VaaSPH-1 to be considered as a guideline to synthesize small FVIIIa-binding molecules, potential new generation of anticoagulants.

Keywords

- snake venoms
- haemostasis
- coagulation inhibitors
- factor VIII inhibitors
- venous thrombosis

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Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide with more than 30% share of all global deaths (www.who.int/mediacentre/factsheets/fs317/en/). Two of them, deep vein thrombosis and pulmonary embolism, originate from the same pathological process known as venous thromboembolism (VTE) in which the blood clot or thrombus is formed most commonly in the leg vein due to dysfunction of haemostasis. The clot can block the blood flow in the vein causing deep vein thrombosis or it can be displaced from the site of formation in the vein to the heart and pulmonary arteries of the lungs to induce pulmonary embolism.¹ VTE is the third leading cause of CVD-related mortality, after myocardial infarction and stroke.^{2,3} One to three persons out of 1,000 die from VTE every year.⁴ Vein thrombosis is caused by the increased activity of blood coagulation factors resulting in hypercoagulability of blood leading to thrombus formation.⁵ Reasons for dysregulation of haemostasis in veins can be environmental or genetic.^{2,6} Among the former are surgery, trauma, prolonged immobility, decreased cardiac output, malignancy and chronic cigarette smoking.^{2,7} Inherited venous thrombotic disease is characterized by an increased activity of one or more pro-coagulant blood proteins (activated form of blood coagulation factor II [FIIa], FVIIa, FVIIIa, FIXa, FXa, FXIa or plasminogen activator inhibitor-1) and/or a decreased activity of one or more anticoagulant blood proteins (tissue factor [TF] pathway inhibitor, anti-thrombin, activated protein C) and/or the presence of FV Leiden.^{8–10}

Pathological blood coagulation is treated with anticoagulants, which down-regulate the activity of thrombin directly or inhibit its formation. To this end, different forms of heparins and vitamin K antagonists have been traditionally used.^{3,11,12} The main problem of treating patients with these drugs is the induction of excessive bleeding, requiring constant monitoring and dose adjustment. Some of these drugs can also interact with food and other drugs, which may induce additional therapeutic complications. Improvements in VTE therapy came with new oral anticoagulants (NOACs), developed to selectively target proteins in the blood coagulation cascade, for example, thrombin (dabigatran) or FXa (apixaban and rivaroxaban).¹³ The onset and offset of their action is rapid, their dose–response and pharmacological profile are predictable and the interaction with food or drugs is negligible. A serious drawback associated with the NOACs therapy is however a severe bleeding that may occur, as there is no reliable test to monitor their therapeutic dosage as well as no appropriate reversal agent to inhibit their action in the case of overdose.^{14–16}

Coagulation process is divided into three pathways—extrinsic pathway, initiated by binding of FVIIa to extrinsic TF, intrinsic pathway, driven mainly by intravascular proteins, for example, FXIIa, FXIa, FIXa and FVIIIa, and common pathway, where both previous pathways meet, starting at the activation of pro-thrombin by FXa/FVa complex (pro-thrombinase complex) resulting in the formation of fibrin clot as the final product.^{17,18} Considering the

exponential amplification of the coagulation process by sequential proteolytic activation of factors in cascades, an anticoagulant drug targeting an upstream component of the coagulation process, such as TF, will inhibit the process more potently than a drug targeting a component in the final step of the process, for example, thrombin.¹⁹ By gene-knockout experiments in mice, it was demonstrated that blood coagulation factors constituting the extrinsic and the common coagulation pathways are fundamental for haemostasis and life.^{19,20} On the contrary, mice were able to live quite normally without components of the intrinsic blood coagulation pathway,²⁰ which malfunction is however mainly responsible for blood hypercoagulability in veins. Components of the intrinsic blood coagulation pathway have been therefore recognized as very suitable therapeutic targets to treat VTE.^{21–24} Anticoagulant therapy directed towards one or more of these coagulation factors would be much safer for patients. While on one hand, it would prevent pathological hypercoagulability of blood in veins, it would preserve on the other reasonably normal coagulation of blood upon vascular injury.

Several attempts to inhibit components of the intrinsic blood coagulation pathway have already been made. Some of them were directed towards FIXa, a serine proteinase (SP), complexing with its co-factor FVIIIa on the surface of Ca²⁺-decorated anionic phospholipid (PL) membrane in the intrinsic tenase complex.²⁵ The intrinsic tenase complex is a key point of the intrinsic coagulation pathway as it converts FX into its activated form. FIXa has been targeted by monoclonal antibodies (e.g. SB249417),^{26–29} NOACs (e.g. TTP889, which was lacking anti-thrombotic effect in vivo and therefore did not enter clinical trials³⁰) and ribonucleic acid aptamers (e.g. pegnivacogin, which failed in phase 3 clinical trials due to inducing severe allergic reactions in 1% of the patients³¹). Drugs targeting FVIIIa have also been developed, for example, human monoclonal antibody (e.g. TB-402) that partly inhibits FVIIIa and has completed phase II clinical studies in patients with orthopaedic surgery.²⁴ Recombinant human forms of activated protein C (e.g. drotrecogin) and soluble thrombomodulin (e.g. recomodulin and solulin) inactivate or promote the inactivation of FVa and FVIIIa; however, none of these drugs have reached the phase III clinical testing.³² The main problem using these proteins in therapy is their immunogenicity.³³ Peptides, analogous to the FIXa-interacting loop on FVIIIa, have also been investigated as anticoagulants³⁴ but there have been no reports about clinical studies using these peptides so far.

Over the past decades, snake venom (SV) components have been extensively investigated as substances for diagnosis and treatment of human diseases.³⁵ In venoms of snakes *Protobothrops flavoviridis* (formerly *Trimeresurus flavoviridis*), *Echis carinatus leucogaster* and *Deinagkistrodon acutus* (formerly *Agkistrodon acutus*), FIX-binding C-type lectin-like proteins have been discovered. None of them has entered pre-clinical or clinical trials yet.^{36,37} *Vipera ammodytes ammodytes* (Vaa) is the most venomous snake in Europe. The most pronounced effect of its venom on a human victim is the effect on its haemostatic system. The

venom contains proteins that disturb the blood coagulation system at different points, inducing pro- or anticoagulant effects.³⁸ In this venom, we have also detected a glycoprotein without an enzymatic activity, which significantly prolonged the activated partial thromboplastin time (aPTT) in human plasma, indicating its effect on the intrinsic blood coagulation pathway. Here, we report purification and detail characterization of this unique and very promising candidate to design, based on its structure, a novel type of selective intrinsic tenase complex inhibitors of therapeutic relevance in the VTE therapy.

Materials and Methods

Purification of Vaa SP Homolog 1

The crude Vaa venom was obtained from the Institute of Immunology, Zagreb, Croatia. Lyophilized venom was kept at -20°C . For the analysis, it was dissolved in 50 mM Tris, 2 mM CaCl_2 , 300 mM NaCl and pH 7.0 (buffer A). The whole purification procedure was performed in a cold room (4°C). Note that 250 μL of the raw venom solution (1 g) was applied on a Sephacryl S-200 Superfine column ($100 \times 4 \text{ cm}$) (GE Healthcare BioSciences AB, Sweden), equilibrated with 15 mL buffer A. Gel chromatography was performed at a constant flow rate of 33.6 mL/h. Concentration of proteins in the mobile phase was monitored by measuring the absorbance at 280 nm (A_{280}). The B1 fraction was dialysed against 20 mM MES, 2 mM CaCl_2 , pH 6.5 (buffer B) and further fractionated on a Mono S 5/50 GL column (GE Healthcare Life-Science, Sweden). Column-retained material was eluted by linear gradient of NaCl from 0 to 1 M in the buffer B. VaaSPH-1 was eluted from the column, concentrated and dialysed against buffer B.

Native Polyacrylamide Gel Electrophoresis

Vaa SP homolog 1 (VaaSPH-1) (2.86 μg) was mixed with one of the coagulation factors (FII(a), FVa, FVII(a), FIX(a), FX(a), FXI(a), FXII(a) and kallikrein) (Haematologic Technologies, Inc., United States) in 1:1, 1:2 or 1:5 molar ratio. FVIII (Recombinate [Antihemophilic Factor (Recombinant)], Shire, United States) (267 U) was dissolved in 2.67 mL Milli-Q water. As no information about the specific activity of Recombinate was available, the amount of FVIII (0.82 U) that was clearly detectable in gels with PageBlue (Thermo Fisher Scientific, United States) was used in each experiment. FVIII was activated to FVIIIa by thrombin (1 nM) in 45-minute incubation at 37°C . FVIIIa was mixed with the increasing amounts of VaaSPH-1 (3, 6, 9 and 15 μg). Mixtures of coagulation factors and VaaSPH-1 were incubated for 5 minutes at 37°C in a pH 6.8 buffer consisting of 62.5 mM Tris/HCl, 25% (m/v) glycerol and 1% (m/v) Bromophenol blue, in which the native structure of the proteins was preserved. Samples were analysed on continuous 10% (m/v) polyacrylamide gels without sodium dodecyl sulphate (SDS), using a running buffer of 25 mM Tris and 192 mM glycine, at 80 to 90 V for 6 hours on ice to limit protein degradation. Proteins in gels were stained with PageBlue.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulphate

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses and silver staining of gels were performed as described in Latinović et al.³⁹ Molecular mass standards were from Fermentas (Thermo Fisher Scientific, Lithuania).

Two-Dimensional Gel Electrophoresis

A total of 9 μL of VaaSPH-1 was analysed per two-dimensional gel electrophoresis as specified,³⁹ the only two differences being in amounts of DeStreak reagent (1.2% [v/v]; GE Healthcare, Sweden) and immobilized pH gradient buffer (0.5% [v/v]; GE Healthcare) added.

Deglycosylation of VaaSPH-1 with Peptide N-glycosidase F

Deglycosylation of 4 μg of dry VaaSPH-1 was accomplished using peptide N-glycosidase F (PNGF, Roche, Germany) essentially as presented in Leonardi et al.⁴⁰

Western Blot Analysis

Western blot (WB) analysis of 2 μg /lane of VaaSPH-1 and 5 μg /lane of Vaa venom was accomplished as described,⁴⁰ only that the nitrocellulose membrane (Serva, Germany) was incubated overnight at 4°C with the rabbit anti-raw Vaa venom serum (anti-Vaa) diluted at 1 to 10,000 in 0.5% (m/v) WB solution (Roche, Germany). Specific bands were visualized on the membrane using the enhanced chemiluminescence (ECL) Lumi-Light^{PLUS} WB substrate (Roche, Germany) on a ChemiDoc MP System gel imager (Bio-Rad, United States). The chemiluminescence signal was detected for 5 minutes using the ChemiDoc MP Chemi Hi Sensitivity application.

Determination of Amino Acid Sequence of VaaSPH-1

N-terminal amino acid sequencing of 4 μg of the isolated native VaaSPH-1 was performed using automated Edman degradation on a Procise 492A Sequencing System (Applied Biosystems, United States). A total of 2 μg of VaaSPH-1 hydrolysate was analysed on an ion trap mass spectrometer 1200 series HPLC-Chip-LC/MSD Trap XCT Ultra (Agilent Technologies, Germany) as described in Leonardi et al.⁴⁰ Deconvolution of mass spectrometry (MS) and tandem MS (MS/MS) spectra is defined in the same reference. Amino acid sequences were compared by alignment using the online software MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>).⁴¹

Proteolytic Activity of VaaSPH-1

The fibrinogenolytic activity of VaaSPH-1 was examined as described,⁴² only the mass ratio 100:1 between the human fibrinogen and VaaSPH-1 was used. The effect of VaaSPH-1 on bovine FX and FIX, pro-thrombin, protein C and plasminogen (Haematologic Technologies, Inc., United States) was tested as follows. Note that 20 μg of each substrate was incubated with or without (control) 1 μg of VaaSPH-1 in 55 μL of 50 mM Tris/HCl, 2 mM CaCl_2 , pH 8.5 at 37°C . A total of 10 μL aliquots of reaction mixtures were withdrawn after 2, 4 and 24 hours and analysed by 12.5% (m/v) SDS-PAGE

under reducing conditions. The gels were stained with PageBlue. FIXa, FXa, α -thrombin and plasmin were used as positive controls.

Thrombin Time, Pro-Thrombin Time and Partial Thromboplastin Time Measurements

To 45 μ L of the normal human plasma in a cuvette, we added 5 μ L of VaaSPH-1 sample in the buffer B with the final concentration of 1 μ M. In the control experiment, 5 μ L of only buffer B was added. Measurements of thrombin time (TT), pro-thrombin time (PT) and aPTT were done as described³⁹ using the BFT II Coagulation Analyser (Siemens, Germany).

Platelet Aggregation/Agglutination Assays

Blood was donated by the experimenter according to permission no. 53/08/11 of the National Medical Ethics Committee of the Republic of Slovenia. Platelet aggregation/agglutination assays were performed in the same way as described by Latinović et al.³⁹ VaaSPH-1 in the final concentration of 1 μ M was used in experiments.

Lupus Anticoagulant Screening and Confirmation Assay

To 75 μ L of normal human plasma in a cuvette, 20 μ L of VaaSPH-1 in buffer B was added with the final concentrations of VaaSPH-1 from 10 to 3,000 nM. In the control experiment, 20 μ L of only buffer B was added to the human plasma. In the case of lupus anticoagulant (LA) screening assay, 95 μ L of LA1 reagent was then added to cuvettes. LA1 reagent (Siemens, Germany) is diluted Russell's viper venom (dRVV), which initiates plasma clotting by direct activation of FV and FX. Confirmation or LA2 reagent (Siemens, Germany) differs from LA1 reagent by containing a high concentration of PLs. Note that 95 μ L of LA2 reagent was added to the mixture of 75 μ L of normal human plasma and 20 μ L of VaaSPH-1 to obtain final concentrations from 10 to 3,000 nM. The BFT II Coagulation Analyser (Siemens, Germany) was used to measure clotting time.

Thrombin Generation Assay

Generation of thrombin in human plasma was measured fluorimetrically using Technothrombin thrombin generation assay (TGA) reagents (Technoclone, GmbH, Austria). A total of 40 μ L of the plasma (TGA control high, lyophilized) were pre-incubated with 4 μ L of VaaSPH-1 (770 nM final concentration) or 4 μ L of Milli-Q water (control) for 2 minutes. Coagulation cascade was then activated by the addition of 10 μ L of TF and PLs (TGA reagent C low) followed by 50 μ L of a fluorogenic substrate for thrombin (TGA substrate). Fluorescence was monitored at 37°C in 1-minute intervals for 90 minutes using a Victor X2 multi-label plate reader (Perkin Elmer, Finland) with the excitation at 355 nm and the emission at 460 nm. Technoclone software (www.technoclone.com) was used to determine the concentration of thrombin at the peak of its generation in nM. The area under the concentration curve corresponds to endogenous thrombin potential. By using less than 5 pM TF in the assay, both the intrinsic and the extrinsic blood coagulation pathways were activated to generate thrombin^{43,44}—therefore, the influence

of VaaSPH-1 on the whole coagulation cascade's thrombin potential was observed.

Pro-Thrombinase Interference Assay

The assay was performed in buffer E (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 140 mM NaCl, 5 mM CaCl₂, 1 mg/mL bovine serum albumin [BSA], pH 7.4) at 37°C. Solution of VaaSPH-1, in different concentrations, was pipetted to 96-well microtiter plates, 25 μ L in each well. Separately, pro-thrombinase complex was constituted by mixing 25 μ L of 10 pM FXa, 25 μ L of 1 nM FVa and 25 μ L of buffer E containing 67 μ M of reconstituted phosphatidylserine:phosphatidylcholine (PC:PS) vesicles (PC:PS in molar ratio 7:3; Avanti Polar Lipids, Alabama, United States). Then the solution containing the pro-thrombinase complex was added to VaaSPH-1 solution in microtiter plates and incubated for 15 minutes. Thereafter, 25 μ L of 12.5 nM pro-thrombin was added and incubated for another 15 minutes. The reaction was quenched with 25 μ L of buffer E supplemented with 50 mM ethylenediaminetetraacetic acid (EDTA). The activity of thrombin, generated by the activity of the pro-thrombinase complex, was assessed using a microplate reader (Tecan, Switzerland) after the addition of 50 μ L of 250 μ M S-2238 and following the absorption at 405 nm (A_{405}). The velocity of substrate hydrolysis was calculated from the absorption curve. The activity of thrombin formed in the absence of VaaSPH-1 was considered 100% (0% inhibition). Percentages of inhibition of the thrombin formation by different concentrations of VaaSPH-1 were calculated using the formula: % inhibition = $\frac{(\text{velocity in the absence of [VaaSPH-1]} - \text{velocity in the presence of [VaaSPH-1]})}{\text{velocity in the absence of [VaaSPH-1]}} \times 100$.

Intrinsic Tenase Interference Assay

The assay was performed in buffer E with 67 μ M PC:PS vesicles (PC:PS in molar ratio 7:3; Avanti Polar Lipids) at 37°C. Note that 25 μ L of FVIII (final concentration of 25 nM) was added to each well of the 96-well microtiter plate. Thirty-minute incubation of FVIII with 25 μ L of 1 nM thrombin resulted in the activation of FVIII. The activation was quenched with the addition of 25 μ L of thrombin inhibitor hirudin (115 U/mL). After the addition of 25 μ L of 2 nM FIXa to each well, the intrinsic tenase complex was formed. Then, to each well 25 μ L of VaaSPH-1 solution (of different concentrations) was added and incubated for 10 minutes (experimental setup 1). In experimental setup 2, additions of FIXa and VaaSPH-1 were reversed. Note that 25 μ L of 50 nM FX was pipetted into each well. Activation of FX to FXa by intrinsic tenase proceeded for 15 minutes at 37°C after which the reaction was terminated by the addition of 25 μ L of buffer E supplemented with 50 mM EDTA. Activity of the generated FXa was assessed after the addition of 25 μ L of 2 μ M synthetic FXa substrate S-2765 and measurement of A_{405} on a microplate reader (Tecan, Switzerland). The velocity of substrate hydrolysis was calculated from the absorption curve. The activity of FXa formed in the absence of VaaSPH-1 was considered 100% (0% inhibition). Percentages of inhibition of FXa formation by different concentrations of VaaSPH-1 were calculated using the formula: % inhibition

= ((velocity in the absence of [VaaSPH-1] - velocity in the presence of [VaaSPH-1]) / velocity in the absence of [VaaSPH-1]) × 100.

FIXa Amidolytic Activity Interference Assay

Note that 30 µL of VaaSPH-1 in 50 mM Tris, 5 mM CaCl₂, 140 mM NaCl, 1 mg/mL BSA and pH 7.4 (spanning concentration range of 0.1–10 µM) and 30 µL of FIX (final concentration of 1 µM), were added to each well of the 96-well microtiter plate and incubated for 10 minutes at 37°C. Then, FIX was activated for 10 minutes by 30 µL of FIXa (final concentration 1 nM). Amidolytic activity of FIXa was assessed by measuring A₄₀₅ on microplate reader (Tecan, Switzerland) after the addition of a chromogenic substrate, 100 µL of 2 mM Spectrozyme (American Diagnostics, United States). The velocity of substrate hydrolysis was calculated from the absorption curve. The activity of FIXa formed in the absence of VaaSPH-1 was considered as 100%.

Structure Modelling, Docking and Molecular Dynamics

Ab initio VaaSPH-1 secondary structure prediction was performed using ROSETTA-based Robetta web server (<http://rosetta.bakerlab.org>).⁴⁵

To identify the most suitable template structure, a blast of Protein Data Bank (PDB) was performed with the VaaSPH-1 sequence. The most homologous sequence to the sequence of VaaSPH-1 found was a sequence of a glycosylated thrombin-like enzyme AHV_{TL-I} from the venom of *Gloydus halys*.⁴⁶ The three-dimensional (3D) structure of this protein (PDB ID: 4E7N) served as a template to model the 3D structure of VaaSPH-1. VaaSPH-1 sequence was aligned with the sequence of the template molecule using Clustal (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The alignment was adjusted manually, taking into account the Robetta secondary structure prediction. The VaaSPH-1 model was generated using the homology modelling program Modeller 9v14 (<http://www.sallilab.org/modeller/>).⁴⁷ Modelling was performed using the 'allHModel' protocol to include hydrogen atoms and default parameters, thorough molecular dynamics (MD) optimization and refinement protocol. The secondary structure of individual loops was re-built using the 'loopmodel' protocol.⁴⁸ The model obtained was further optimized using the NAMD 2.11 (<http://www.ks.uiuc.edu/Research/namd/>) MD plug-in in the VMD v1.9.2.27 program (<http://www.ks.uiuc.edu/Research/vmd/>) by simulating movements in water. MD

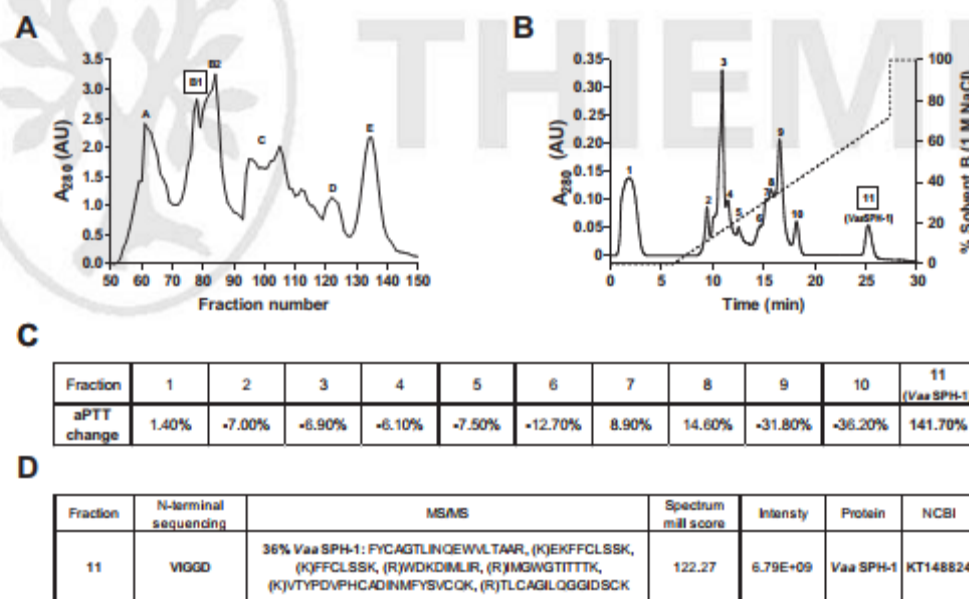


Fig. 1 Purification of *Vipera ammodytes ammodytes* serine proteinase homolog 1 (VaaSPH-1) from the Vaa venom. Size-exclusion chromatography of the crude Vaa venom was accomplished on a Sephacryl S-200 superfine column. The elution of proteins was monitored at 280 nm (A). The fraction B1 was pooled and sub-fractionated on the ion exchange chromatography on Mono S 5/50 GL column (B). Column-retained proteins were eluted by a linear gradient of NaCl as depicted. Eleven fractions were collected and tested for their effect on blood coagulation. The effect of fractions on activated partial thromboplastin time (aPTT) is shown (C). A pronounced anticoagulant effect was exhibited by fraction 11. Structural characterization, N-terminal amino acid sequencing and tandem mass spectrometry (MS/MS) of the protein in fraction 11 revealed it as a transcript of a gene, which complete complementary deoxyribonucleic acid (cDNA) sequence is deposited under the National Center for Biotechnology Information (NCBI) accession number KT148824 (D), corresponding to a SP-like protein harbouring two mutations in its active site (framed blue in Fig. 2). We termed it therefore a homologue of SP from Vaa or VaaSPH-1.

simulations were performed from 0 to 20 ps. Obtained structures were evaluated in PyMol and with SWISS MODEL (http://swissmodel.expasy.org/workspace/?func=tools_structureassessment1).

To get insight into the atomic details of interaction between VaaSPH-1 and FVIIIa in the intrinsic tenase complex, 3D structures of FIXa and VaaSPH-1 were swapped in the model (1 μ s extraction of the MD simulation) of human intrinsic tenase complex.²⁵ FIXa consists of four structural domains, SP, γ -carboxyglutamic acid-rich (GLA) and two epidermal growth factor (EGF)-like domains (EGF1 and EGF2). Docking and energy calculations were performed with the web server version of High Ambiguity Driven protein-protein Docking (HADDOCK) modelling program with all of the parameters set to default values, using a refinement interface and finally analysed in PyMol.

Results

Purification and Basic Biochemical Characterization of VaaSPH-1

Vaa venom was size-fractionated by gel chromatography (Sephacryl S-200 Superfine column) into several peaks (~Fig. 1A). The B1 fraction, containing proteases with molecular masses in the range between 30 and 40 kDa,³⁸ was further separated on cation exchange chromatography (~Fig. 1B). Proteins retained by the ion exchanger were eluted gradually from the column by increasing the content

of salt in the eluting buffer. Eleven peaks were collected, dialyzed and tested for their effect on blood plasma. All fractions exhibited either pro- or anticoagulant effect (~Fig. 1C). Fraction 11, eluted as the last of all, especially attracted our attention for its pronounced anticoagulant effect (~Fig. 1C). The fraction was homogenous as confirmed by the N-terminal amino acid sequencing, which result was a single signal corresponding to the VIGGD sequence. Using MS/MS, we determined its partial amino acid sequence (~Fig. 1D) and found it identical to the amino acid sequence translated from the Vaa venom gland complementary deoxyribonucleic acid library clone deposited under the National Center for Biotechnology Information (NCBI) accession number KT148824 (~Fig. 2). Experimentally, we covered 36% of the predicted mature part of the amino acid sequence translated from the KT148824 nucleotide sequence. Protein corresponding to the KT148824 nucleotide sequence is structurally a SP but two of three canonical SP active site amino acid residues are mutated in this molecule (framed blue in ~Fig. 2), so it should not be proteolytically active. Indeed, as we have demonstrated (~Fig. 3D), the protein in fraction 11 was not proteolytically active. For this reason, we termed it VaaSPH-1.

VaaSPH-1 displayed multiple isoelectric points (pIs) (~Fig. 3A), which is typical for glycosylated proteins. When VaaSPH-1 was treated with PNGF, its molecular mass dropped from 35 to 29 kDa, indicating that N-glycans represent 6 kDa of VaaSPH-1 molecular mass (~Fig. 3B). VaaSPH-1 appeared in seven glycoforms with pIs spanning from 6.0 to 9.5. Two most

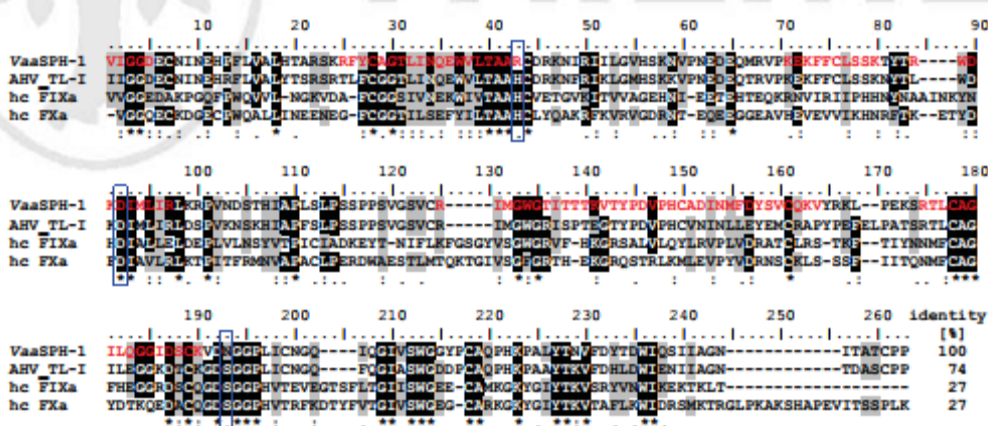


Fig. 2 Amino acid sequence of *Vipera ammodytes ammodytes* serine proteinase homolog 1 (VaaSPH-1) and its comparison with some relevant SPs. The complete primary structure of VaaSPH-1 was inferred from its complementary deoxyribonucleic acid (cDNA) sequence deposited at National Center for Biotechnology Information (NCBI) (KT148824). Red letters indicate parts of the VaaSPH-1 sequence that were experimentally determined (altogether 36% of the mature protein). The amino acid sequence of VaaSPH-1 is aligned with AHV_{TL-I}, a thrombin-like enzyme from the venom of *Gloydus halys*, the heavy chain (hc) of factor IXa (FIXa) and the heavy chain of FXa. The extent of the primary structure identity with VaaSPH-1 is indicated. The positions of three active site amino acid residues in SPs are framed blue. In proteolytically active SPs, these positions are occupied by His, Asp and Ser, while in VaaSPH-1 His and Ser are exchanged for Arg and Asn. Amino acid residues present at the same position in all sequences are printed on the black background and designated by an asterisk (*) in the row below all sequences. Positions in sequences harbouring conservative substitutions are grey backgrounded and designated by a colon (:) in the row below all sequences, while positions of semi-conservative and non-conservative substitutions are white backgrounded and marked by (.) below the sequences.

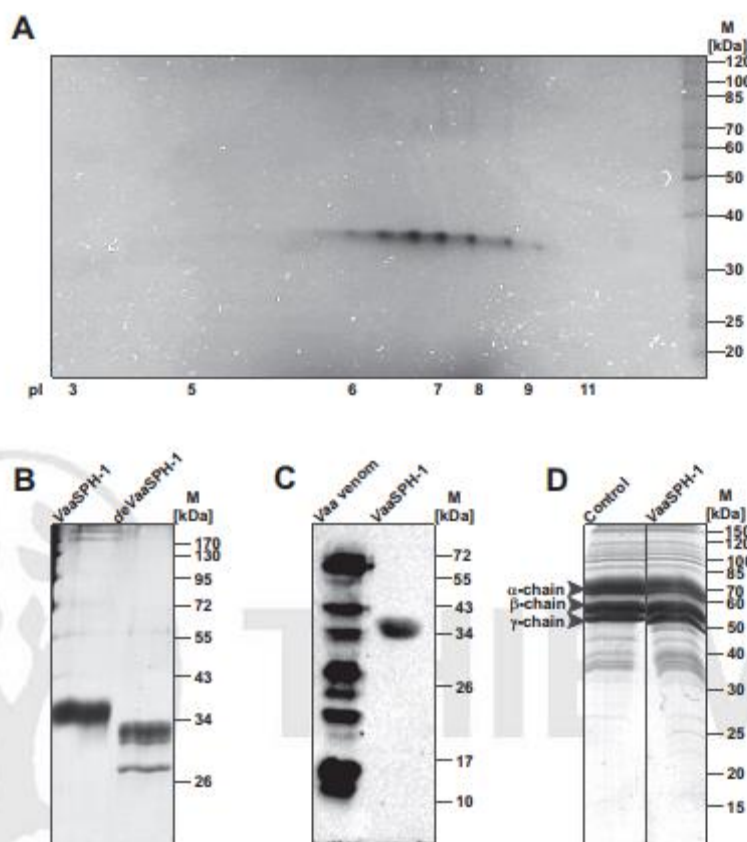


Fig. 3. Basic biochemical characteristics of *Vipera ammodytes ammodytes* serine proteinase homolog 1 (VaaSPH-1). (A) Isoelectric focusing of VaaSPH-1 was accomplished using pH 3–11 immobilized pH gradient (IPG) strips, in the first, and the reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% (w/v) polyacrylamide gel, in the second dimension. Silver staining revealed that VaaSPH-1 focused in multiple points ranging from 6.0 to 9.5, characteristic for glycoproteins. (B) VaaSPH-1 was incubated with peptide-N-glycosidase F at 37°C overnight and then analysed on SDS-PAGE as above. A drop of molecular mass from 35 to 29 kDa specifies VaaSPH-1 as an N-glycosylated protein. (C) VaaSPH-1 was separated by SDS-PAGE under reducing conditions and electrotransferred onto a nitrocellulose membrane. VaaSPH-1 was strongly immunostained using anti-Vaa serum, horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin Gs (IgGs) and the enhanced chemiluminescence (ECL) detection system. (D) The absence of proteolytic activity of VaaSPH-1 as demonstrated on human fibrinogen. The substrate was incubated with VaaSPH-1 at the mass ratio 100:1. In the control experiment, no VaaSPH-1 was added. Following the 60-minute incubation at 37°C, the samples were analysed on SDS-PAGE under reducing conditions and stained with PageBlue. Positions of fibrinogen α , β and γ chains are indicated.

abundant glycoforms have pI of 6.8 and 7.1. From the primary structure, two possible N-glycosylation sites were inferred in VaaSPH-1, Asn123 and Asn253. Pure VaaSPH-1 was highly immunodetectable by rabbit anti-Vaa venom serum (– Fig. 3C). According to the amino acid sequence, VaaSPH-1 is an SP. However, two amino acid residues out of three, constituting the canonical catalytic triad in SPs, are mutated in VaaSPH-1. As shown in – Fig. 2, in active SPs from SVs the essential catalytic residues are usually Ser195, His57 and Asp102 (chymotrypsin numbering). In VaaSPH-1, only Asp102 is present while the other two are Asn195 and Arg57, which is the obvious reason for the absence of its

proteolytic activity. VaaSPH-1 was not able to hydrolyse any protein substrate tested in vitro, either pro-thrombin, FIX, FX, protein C, plasminogen or fibrinogen. As demonstration, the negative result obtained with the latter is displayed in – Fig. 3D.

VaaSPH-1 Inhibits Blood Coagulation without Affecting Platelet Function

To explain the pronounced anticoagulant effect of VaaSPH-1 mechanistically (– Fig. 1C and –Supplementary Fig. S1, available in the online version), we first inspected its interference with platelets. As shown in – Fig. 4A, VaaSPH-1 did

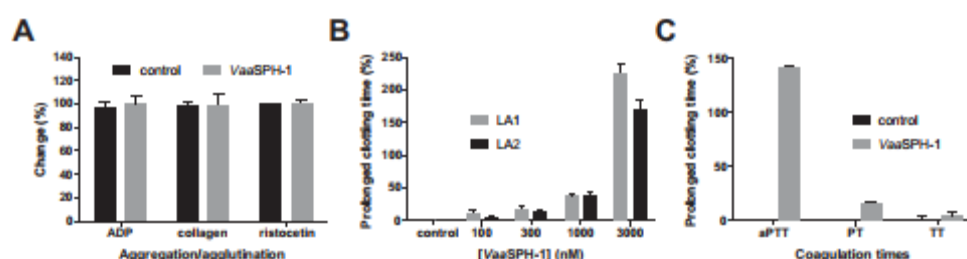


Fig. 4 *Vipera ammodytes ammodytes* serine proteinase homolog 1 (VaaSPH-1) anticoagulant activity is not platelet-directed. (A) We assessed the influence of VaaSPH-1 on platelet aggregation/agglutination by pre-incubating platelet-rich plasma with 1 μ M VaaSPH-1 at 37°C. Then an agonist of platelet aggregation, adenosine diphosphate (ADP) or collagen, or platelet agglutination, ristocetin, was added to the platelet solution and its optical density measured. Control values, taken as 100% change in optical density, were obtained in the absence of the venom protein. As evident, VaaSPH-1 does not affect the platelet function. (B) By diluted Russell's viper venom (dRVV) we activated factor V (FV) and FX in human blood plasma to constitute a pro-thrombinase complex on negatively charged phospholipids (PLs) of the plasma membrane of platelets. The complex then activates pro-thrombin to induce blood coagulation (lupus anticoagulant [LA]1 assay). We measured the difference in time for blood to coagulate in the absence and in the presence of the indicated concentration of VaaSPH-1 and expressed the difference in per cents of the clotting time obtained without the addition of VaaSPH-1. The LA2 assay was performed in the same way as the LA1 assay just in the presence of an excess of negatively charged PLs to see whether the anticoagulant effect of the venom component stems from its interference with the PLs or not. Even at the very high concentrations of VaaSPH-1, the ratio between LA1 and LA2 result does not exceed 1.4, indicating that the negatively charged PLs are not the main target of VaaSPH-1. (C) The effect of 1 μ M VaaSPH-1 on blood coagulation was analysed using three clinical blood coagulation assays: pro-thrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT). In control experiments, blood-clotting times were measured in the absence of VaaSPH-1. VaaSPH-1 exhibited a distinct effect on the aPTT, prolonging it for 140% according to the control value. All experiments were performed in duplicates and results are displayed as mean $\pm$ standard deviation (SD).

not affect aggregation and agglutination of platelets when tested *in vitro* using collagen, adenosine diphosphate and ristocetin as platelet agonists.

However, as we demonstrated a selective binding of VaaSPH-1 to the negatively charged PLs, of the highest avidity for PS (–Supplementary Fig. S2, available in the online version), a possibility emerged that this is the trait defining VaaSPH-1 as an anticoagulant. It is known that complexes of coagulation factors, for example, the pro-thrombinase complex, constitute and function on the negatively charged PL surfaces of the plasma membrane of platelets. High affinity binding of VaaSPH-1 to such PLs could interfere with the formation of these complexes and consequently with the blood coagulation process. To verify this assumption, we performed the LA assays in the presence of increasing concentrations of VaaSPH-1. In the LA1 assay, dRVV was added to the human plasma to produce FVa and FXa, which then associated with the negatively charged surface of platelets to form pro-thrombinase. This cleaved pro-thrombin to thrombin, which induced blood coagulation that we observed. As evident from –Fig. 4B, addition of VaaSPH-1 to the plasma prolonged the time for plasma to coagulate. The effect was dose-dependent and may be the result of the interaction of VaaSPH-1 with the protein components of the pro-thrombinase complex or the negatively charged PLs. The ambiguity was resolved by performing the LA2 assay, which differs from the LA1 assay only for the presence of an excess of the negatively charged PLs. As we have established earlier, the activity of thrombin was practically abolished in the presence of sub-micromolar concentrations of VaaSPH-1 (–Supplementary Fig. S1, available in the online version) rendering the blood clinically incoagulable. However, the

results of LA2 and LA1 assays were largely similar (–Fig. 4B) at concentrations up to 3 μ M, indicating that the binding of VaaSPH-1 to the negatively charged PLs is not the main contributor to the inhibition of pro-thrombinase complex. This primarily stems from its interference with FVa or FXa or both. VaaSPH-1 therefore does not affect platelet function to inhibit blood coagulation but does this by acting on certain protein factors in the coagulation cascade.

VaaSPH-1 Binds Multiple Coagulation Factors

After demonstrating that the anticoagulant effect of VaaSPH-1 is mainly the result of its interference with protein factors in the blood coagulation cascade, we focused on identification of the factors involved.

Initially, we tested the influence of VaaSPH-1 on three blood coagulation times, aPTT, PT and TT (–Fig. 4C). In human plasma, 1 μ M VaaSPH-1 exerted a pronounced effect on the aPTT, prolonging it for 140%. Much less, for approximately 20%, it also extended the PT, but it showed no effect on the TT (–Fig. 4C). As evident from –Fig. 4B, 1 μ M VaaSPH-1 prolonged clotting time by approximately 45% in the LA assays, in which FV and FX were directly activated by dRVV. Consolidating these results, VaaSPH-1 targets pro-thrombinase complex but also affects components of the intrinsic and the extrinsic pathway of blood coagulation. However, the SV protein does not affect the activation of fibrinogen by thrombin.

Next, we assessed the affinity of VaaSPH-1 for different blood coagulation factors using the native PAGE. VaaSPH-1 was incubated with a particular blood coagulation factor after which the mixture was electrophoresed under non-denaturing conditions to reveal whether both proteins

formed the complex or not. By detecting a protein band on a different position in a gel than positions characteristic for VaaSPH-1 and coagulation factor alone, we concluded that VaaSPH-1 formed a complex with pro-thrombin, FVa, FVII and FVIII (depicted as FVII(a)), FVIIIa, FIX(a) and FX(a). Some analyses are displayed in ~Fig. 5A–C, the others can be seen in ~Supplementary Fig. S3A (available in the online version). If the molar ratio between VaaSPH-1 and any of these factors was increased, more factor was complexed and the band corresponding to a complex became more intensive (~Fig. 5A). The native PAGE results also implied that VaaSPH-1 does not associate with FXI(a), FXII(a) (~Fig. 5D), kallikrein and thrombin (~Supplementary Fig. S3B, available in the online version). VaaSPH-1 interfered

therefore with the GLA-domain-containing blood coagulation factors implying its interaction with the GLA domain, and the co-factors, FVa and FVIIIa. Noteworthy is that binding of VaaSPH-1 to coagulation factors did not abolish their hydrolysis of small chromogen substrates (e.g. FIXa in ~Fig. 6D; other results are not shown).

VaaSPH-1 Inhibits Activities of Blood Coagulation Complexes

To verify the hypothesis that VaaSPH-1 exerts its anticoagulant activity also by obstructing the functions of the intrinsic tenase and pro-thrombinase, two important complexes in the blood coagulation cascade, we assembled these complexes in vitro and measured their activity in the presence of

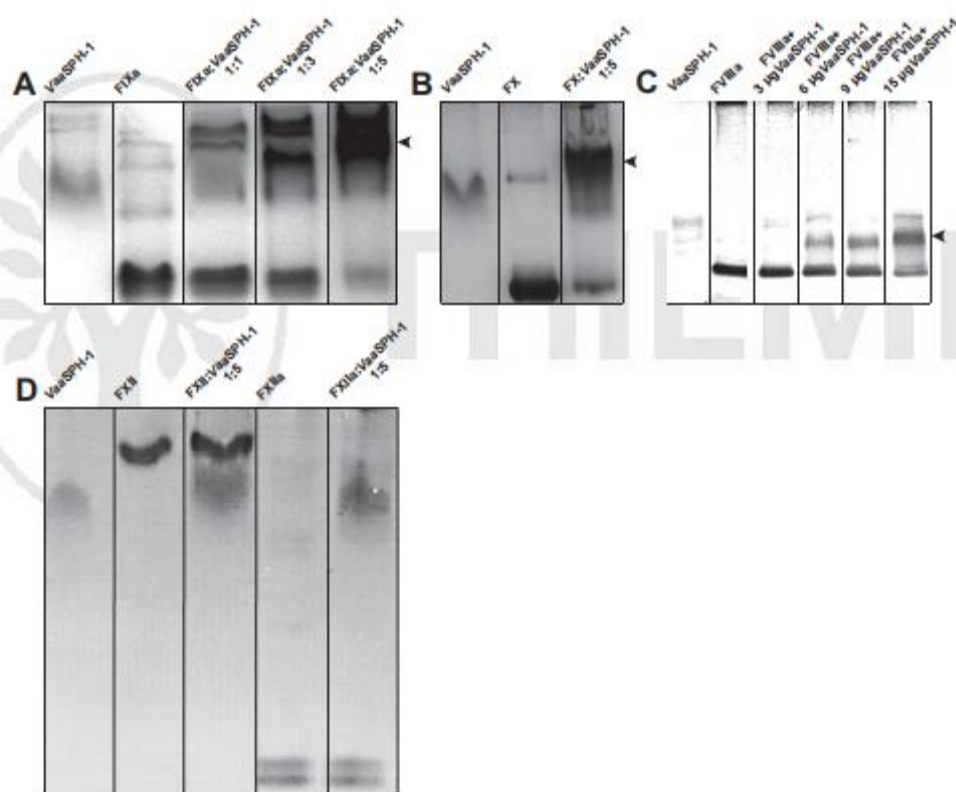


Fig. 5 *Vipera ammodytes ammodytes* serine proteinase homolog 1 (VaaSPH-1) binds to different coagulation factors. Each blood coagulation factor was mixed with VaaSPH-1 in the indicated molar ratio, and incubated at 37°C and pH 6.8 for 5 minutes. Samples were analysed by polyacrylamide gel electrophoresis (PAGE) on continuous 10% (m/v) polyacrylamide gels under non-denaturing conditions to analyse whether VaaSPH-1 and a coagulation factor form the complex or not. Lanes in which only VaaSPH-1 or a coagulation factor was electrophoresed serve to define positions of non-complexed proteins. In panel (A), complexing of factor IXa (FIXa) and VaaSPH-1 is demonstrated. By increasing the molar ratio between both proteins, from 1:1 to 1:5, more complex was formed. (B) FX and VaaSPH-1 associated under used experimental conditions. (C) The complex also formed between FVIIIa and VaaSPH-1. Raising the ratio of VaaSPH-1 in the mixture increased complexing of the two molecules. (D) Some blood coagulation factors, for example, FXII and FXIIa, did not form a complex with VaaSPH-1. This is indicated by the fact no additional protein band occurred upon analysis of the mixture, besides the two bands corresponding to VaaSPH-1 and FXII(a) as specified in the reference lanes. Gels were stained with PageBlue.

the increasing concentrations of VaaSPH-1 (~Fig. 6). Under experimental conditions used, VaaSPH-1 was able to completely inhibit the activity of the pro-thrombinase complex at concentrations above 0.7 μ M (~Fig. 6A). The half-maximal inhibition of pro-thrombinase activity was reached at 164.1 nM VaaSPH-1 (IC_{50}). VaaSPH-1 also inhibited the activity of the intrinsic tenase complex. The complex was constituted on the negatively charged PLs, first, by forming FVIIIa from FVIII in situ using thrombin. Thrombin activity was quenched by hirudin and then FIXa was added. Such mixture, containing the intrinsic tenase complex, was incu-

bated with the increasing concentrations of VaaSPH-1 and then the remaining FX-activating ability was assessed (~Fig. 6B). VaaSPH-1 inhibited the intrinsic tenase activity, reaching approximately 60% inhibition at 2 μ M. The absence of complete inhibition at such a high concentration of VaaSPH-1 may be due to experimental setup, for example, the presence of excessive SP inhibitor hirudin. VaaSPH-1 inhibits in vitro also the activity of the extrinsic tenase complex (~Supplementary Fig. S4, available in the online version), but its much more pronounced effect on aPTT than on PT implies that the hallmark of the anticoagulant activity

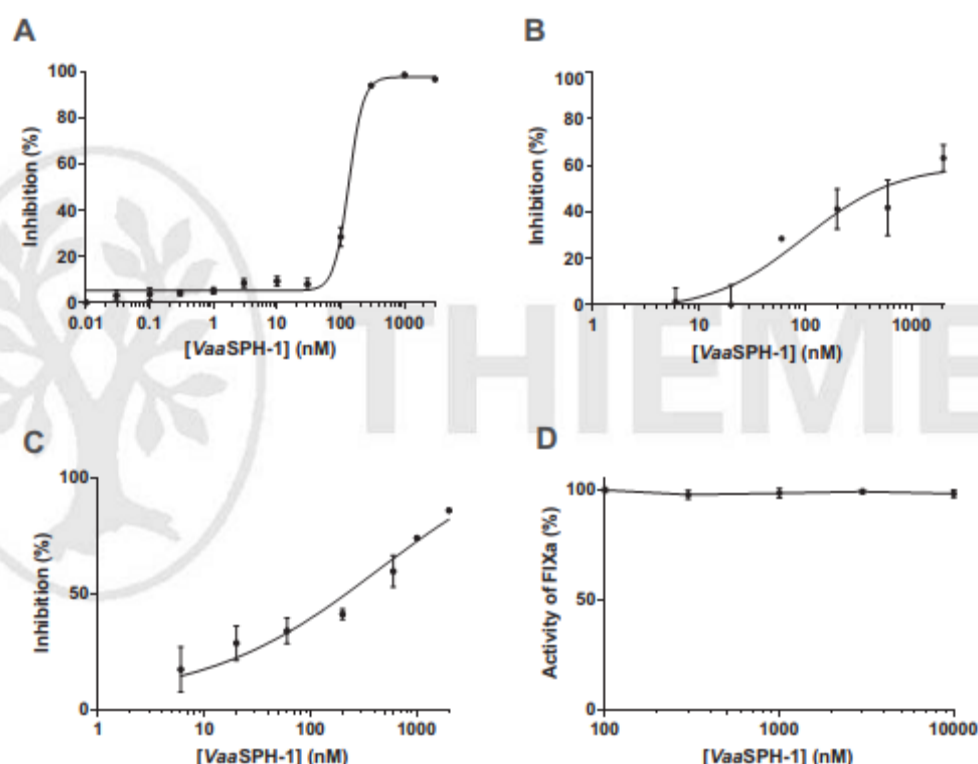


Fig. 6 *Vipera ammodytes ammodytes* serine proteinase homolog 1 (VaaSPH-1) inhibits pro-thrombinase and intrinsic tenase but not factor IXa (FIXa) activity. (A) FIXa and FVa were assembled into the pro-thrombinase complex on phosphatidylserine/phosphatidylcholine (PC/PS) lipid vesicles in vitro at pH 7.4 and 37°C. To the complex, the increasing concentrations of VaaSPH-1 were added, incubated for 15 minutes and then its substrate, prothrombin, was added for 15 minutes. The activity of pro-thrombinase-generated thrombin was assessed using a chromogenic substrate S-2238, following the absorption at 405 nm (A_{405}). (B) FVIII was mixed with PC/PS lipid vesicles and activated by thrombin at pH 7.4 and 37°C until the addition of its inhibitor, hirudin, 30 minutes later. Intrinsic tenase complex formed upon the addition of FIXa (setup 1). To the complex, increasing concentrations of VaaSPH-1 were added and incubated for 10 minutes, followed by the addition of its substrate, FX, and incubation for another 15 minutes. The activity of tenase-generated FIXa was determined using a chromogenic substrate S-2765 and by measuring A_{405} . (C) Besides the reversed order of addition of FIXa and VaaSPH-1 (setup 2), all other conditions were identical to those in setup 1. (D) FIX was mixed with VaaSPH-1 and incubated for 10 minutes at pH 7.4 and 37°C. FIX was activated in situ by addition of FIXa. The activity of FIXa formed in the presence of VaaSPH-1 is considered as 100%. In all three interference assays, percentages of inhibition by different concentrations of VaaSPH-1 were calculated using the formula: % inhibition = $\left[\frac{\text{reaction velocity in the absence of [VaaSPH-1]} - \text{reaction velocity in the presence of [VaaSPH-1]}}{\text{reaction velocity in the absence of [VaaSPH-1]}} \right] \times 100$. All experiments were performed in duplicates and results are displayed as mean $\pm$ standard deviation (SD). Experimental details can be found in the 'Materials and Methods' section.

of VaaSPH-1 is the inhibition of the pro-thrombinase and the intrinsic tenase activities.

VaaSPH-1 Inhibits the Activity of Intrinsic Tenase Mostly by Binding to FVIIIa

VaaSPH-1 can interact with both constituents of the intrinsic tenase complex, FIXa and FVIIIa, as well as with its substrate FX (~Fig. 5A and C). To expose the most relevant interaction of VaaSPH-1 for the inhibition of the intrinsic tenase activity, we measured the hydrolysis of a chromogenic substrate Spectrozyme by FIXa in the presence of increasing concentrations of VaaSPH-1. As evident from ~Fig. 6D, hydrolytic activity of FIXa was not affected by the presence of VaaSPH-1, not even at 10 μ M concentration, meaning that VaaSPH-1 does not obstruct the access of the substrate into the active site of FIXa. Such result suggests that VaaSPH-1 inhibits the activity of intrinsic tenase by binding to FVIIIa in place of FIXa.

We tested this idea by performing the intrinsic tenase interference assay in alternative way (setup 2). Differently as in setup 1 (~Fig. 6B), where the complex was formed first and then VaaSPH-1 added, in setup 2, FIXa was added last. If VaaSPH-1 occupies the FIXa binding site on FVIIIa, then, in the latter case, the inhibition should be more effective than in the former case. Confirming our hypothesis, the extent of inhibition of the intrinsic tenase activity was higher at the same concentration of VaaSPH-1 (~Fig. 6C). For example, at 2 μ M VaaSPH-1 (the highest concentration tested), the inhibition was higher for approximately 25% in the case of the experimental setup 2 than in the case of setup 1. Therefore, we can conclude that VaaSPH-1 is inhibiting the activity of the intrinsic tenase complex mainly by binding to FVIIIa.

Structural Insight into the Interaction of VaaSPH-1 with the Intrinsic Tenase Complex

Using molecular modelling approach, we proceeded to get insight into atomic details of the VaaSPH-1 binding to FVIIIa. To this end, we first constructed a 3D model of VaaSPH-1. Next, we replaced the structure of FIXa in the intrinsic tenase complex²⁵ with the VaaSPH-1 model and docked it with the FVIIIa molecule.

Modelling of VaaSPH-1 3D Structure

3D model of VaaSPH-1 (~Fig. 7B) was built using the crystal structure of thrombin-like enzyme AHV_{TL}-I (AHV_{TL}; PDB ID: 4E7N) from *Gloydius halys* (formerly *Agkistrodon halys*) venom as a template.⁴⁶ Primary structures of both proteins are namely 74% identical and 94% homologous. Comparison of the constructed 3D model of VaaSPH-1 and the crystal structure of AHV_{TL}-I by calculating the root mean square deviation (RMSD) of backbone atomic positions of the two molecules revealed extremely high resemblance. RMSD of only 0.8 Å justified our choice of AHV_{TL}-I as a structural template to model VaaSPH-1.

Docking of VaaSPH-1 into the Intrinsic Tenase Complex
Intrinsic tenase is the complex of FVIIIa and FIXa that constitutes on the platelet plasma membrane. The SP subunit of the complex, FIXa, cleaves the substrate (FX) and

activates it (FXa). By its structure, VaaSPH-1 is a SP, as FIXa and FXa (~Fig. 2). Experimental results suggest that VaaSPH-1 inhibits the intrinsic tenase complex activity by interfering with the binding of FIXa to FVIIIa and not of FX to the formed complex. We verified such suggestion also in silico by comparison of our 3D model of VaaSPH-1 with the crystal structures of the heavy chain (hc) of human FIXa⁴⁹ and hc FX.⁵⁰ RMSD values calculated for respective pairs of protein structures were 1.2 and 1.8 Å, additionally supporting our hypothesis that VaaSPH-1 competes with FIXa for binding to FVIIIa.

To get a more detailed insight into the interaction area between VaaSPH-1 and FVIIIa in the intrinsic tenase complex, we replaced the molecule of FIXa for VaaSPH-1 in the model of the human intrinsic tenase complex (~Fig. 7A)²⁵ and performed the docking procedure using HADDOCK program to obtain the energetically optimal structure (~Fig. 7B). Using the same program, we calculated energies (Van der Waals, electrostatic and desolvation energy) for the intrinsic tenase complex containing VaaSPH-1, full-length FIXa or hc FIXa (~Fig. 7C). According to the energy released upon formation of the complex, the complex containing VaaSPH-1 was as stable as the complex containing the full-length FIXa and much more stable than the complex containing the hc FIXa. This means that the interaction of VaaSPH-1, comprising only the SP domain, with FVIIIa is much stronger than the interaction of the FIXa SP domain (i.e. the hc FIXa) with FVIIIa, reaching the strength of the interaction of the full-length FIXa, comprising interactions of its SP, GLA and two EGF domains with the constituents of the intrinsic tenase complex. In particular, VaaSPH-1 forms significantly stronger electrostatic and Van der Waals interactions with FVIIIa than the hc FIXa (~Fig. 7C). Amino acid residues of VaaSPH-1 involved in major electrostatic interactions with FVIIIa are Gln153, Lys154, Arg157, Glu161 and Lys162. In the case of hc FIXa, only Lys160 forms such interaction. Amino acid residues that are also involved in binding to FVIIIa but contribute less to the interaction are Asp148, Ser150, Lys158, Leu159 and Pro160 in the case of VaaSPH-1, and Asp151, Thr154, Leu156, Arg157 and Thr162 in the case of hc FIXa (~Fig. 7A and B).

Discussion

SVs, especially those from the Crotalidae, Elapidae and Viperidae snakes, are known to be rich sources of proteins, which strongly affect blood coagulation.⁵¹ These proteins are usually very specific for their molecular targets in the coagulation system, resistant to physiological inhibitors and stable in vitro and in vivo. Their appropriateness for application in clinical diagnostics and as therapeutics has been demonstrated several times.⁵² SV proteins affecting the coagulation system are members of different protein families – SPs, metalloproteases, C-type lectins, disintegrins and secreted phospholipases A₂. SPs were shown to affect blood coagulation in different ways, either preventing or propagating it.³⁶ Hitherto, anticoagulant SPs have been shown to act as protein C activators and thrombin-like enzymes.⁵²

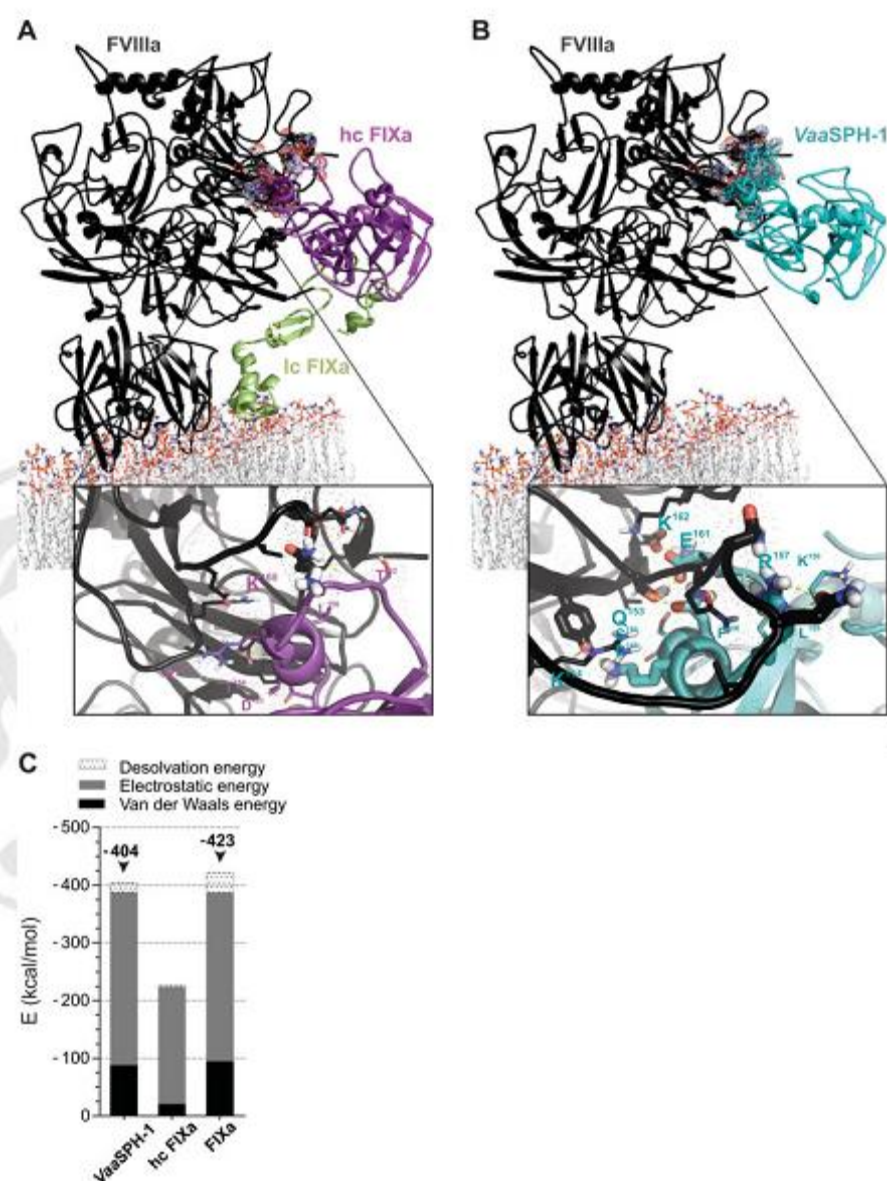


Fig. 7 Docking of *Vipera ammodytes ammodytes* serine proteinase homolog 1 (VaaSPH-1) into the intrinsic tenase complex. (A) The human intrinsic tenase consists of factor DIXa (FIXa), heavy (hc = violet) and light chain (lc = green), and FVIIIa (grey) complexed on the negatively charged plasma membrane of platelet.²⁵ The hc FIXa is comprised of serine proteinase (SP) domain, while the lc FIXa is comprised of γ -carboxyglutamic acid-rich (GLA) and two epidermal growth factor (EGF) domains. FIXa is bound in the complex by interacting with FVIIIa, including all of its domains, and phospholipids (PLs). (B) In the intrinsic tenase complex,²⁵ the atomic coordinates of FIXa were replaced with the co-ordinates of VaaSPH-1 (blue) and docked by High Ambiguity Driven protein-protein Docking (HADDOCK) to generate thermodynamically most optimal structure. Contact areas between FIXa and FVIIIa (A), and VaaSPH-1 and FVIIIa (B) are zoomed in. Amino acid residues involved in major electrostatic interactions are indicated with large letters while those contributing less to the interaction with smaller. (C) Using HADDOCK, Van der Waals, electrostatic and desolvation energies were calculated for the intrinsic tenase complex and complexes containing VaaSPH-1 or hc FIXa instead of FIXa. Although consisting just of the SP domain, VaaSPH-1 forms as stable complex as the full-length FIXa, and substantially more than hc FIXa alone.

Pathological activity of both types of anticoagulant SPs is based on their ability to hydrolyse plasma proteins. Here, we present the first SP family member, whose strong anticoagulant activity is not proteolytic activity-dependent, representing therefore an original molecular platform to develop innovative anticoagulant substances. We purified this protein from the venom of the nose-horned viper and named it VaaSPH-1.

The isolation procedure consisted of two chromatographic steps, a gel filtration and a cation exchange chromatography step (~Fig. 1A and B). VaaSPH-1 eluted from the ion exchanger in fraction 11 and very significantly prolonged aPTT (~Fig. 1C). Its low effect on PT and no effect on TT, suggested a major action of this fraction on the intrinsic pathway of blood coagulation, the feature, which has been intensively sought. Namely, an anticoagulant acting on the intrinsic coagulation pathway would be much safer for medical application²¹—on one hand, it would prevent pathological hypercoagulability of blood in veins, while on the other it would not compromise coagulation of blood upon vascular injury. N-terminal amino acid sequencing and MS/MS analysis of the fraction 11 confirmed VaaSPH-1 as the only protein component present in this fraction (~Fig. 1D). Contrary to our expectation, based on the long retention time of VaaSPH-1 on the strong cation exchange chromatography column (~Fig. 1B), the IEF revealed VaaSPH-1 as essentially neutral protein (~Fig. 3A). Such behaviour indicates existence of an area(s) on the protein surface with higher density of basic amino acid residues. The IEF analysis also revealed that VaaSPH-1 exists in multiple glycoforms. The fact that all these glycoforms, irrespective on their pI, which span from 6.0 to 9.5, eluted in fraction 11, supports our idea of the presence of discrete concentration(s) of the positive charge on the VaaSPH-1 molecule.

Amino acid sequence analysis (~Fig. 1D) revealed VaaSPH-1 as the SP-like protein encoded by the Vaa gene deposited at NCBI under accession number KT148824 (~Fig. 2). Consistent with mutation of two out of the three canonical SP active site amino acid residues (His57 to Arg and Ser195 to Asn, only Asp102 is present⁵³), VaaSPH-1 did not hydrolyse any of the protein substrates tested (e.g. fibrinogen in ~Fig. 3D). VaaSPH-1 is not the first SP-like protein discovered in SVs. Such proteins have been found before⁵⁴ but VaaSPH-1 is the first reported to influence the blood coagulation process. Besides its inhibition of the intrinsic pathway of blood coagulation, the absence of the enzymatic activity is another strong attribute for VaaSPH-1 as a candidate lead compound for development of a new generation of safer anticoagulants.

To employ a molecule as a lead compound, the precise knowledge of its molecular mechanism of pharmacological action is essential. VaaSPH-1 induced a strong anticoagulant effect. In the presence of sub-micromolar concentration of VaaSPH-1, the blood became clinically incoagulable as the formation of thrombin in blood plasma was reduced below the amount necessary for the initiation of fibrin clot formation (~Supplementary Fig. S1, available in the online version), which is approximately 0.2% of its total capacity.⁵⁵

Such a radical drop of thrombin production was observed in blood of patients with severe FVIII deficiency,⁵⁶ representing a hint for the molecular target of VaaSPH-1 in the blood.

According to its retention on the cation exchange column (~Fig. 1B), VaaSPH-1 behaves as a basic SP, and basic SPs usually display direct platelet aggregating activity.⁵⁷ This was not the case with VaaSPH-1. The anticoagulant activity of VaaSPH-1 was not due to its inhibition of platelet coagulation functions (~Fig. 4A). Negatively charged PL domains on the plasma membrane of platelets are platforms for assembly of blood coagulation complexes, tenase and pro-thrombinase,^{58,59} and their optimal function in production of thrombin.^{60–62} In spite of its preference for the negatively charged PLs, in particular PS (~Supplementary Fig. S2, available in the online version), blood-clotting tests (LA1 and LA2) to diagnose PL-related abnormalities did not confirm that VaaSPH-1 anticoagulant activity originates from its interference with PLs (~Fig. 4B). Obviously, this stems from the interference of VaaSPH-1 with one or more protein components of the coagulation cascade (~Fig. 5), as suggested by its major and dose-dependent influence on the aPTT (~Fig. 4C), particularly with coagulation factor(s) of the intrinsic pathway of coagulation. The native PAGE analysis did not confirm interactions between VaaSPH-1 and FXI(a), FXII(a) and kallikrein (~Fig. 5D and ~Supplementary Fig. S3B, available in the online version), that is, blood coagulation factors of the intrinsic coagulation pathway upstream the intrinsic tenase complex, but clearly demonstrated the association of VaaSPH-1 with FVIIIa and FIX(a) (~Fig. 5A and C, and ~Supplementary Fig. S3A, available in the online version). This exposed the interaction of VaaSPH-1 with intrinsic tenase complex as a factor in its anticoagulant action. Explaining the effect of VaaSPH-1 on PT (~Fig. 4C), the native PAGE analysis confirmed also interactions of VaaSPH-1 with FVII(a), FX(a), FV(a) and pro-thrombin (~Fig. 5B and ~Supplementary Fig. S3A, available in the online version), constituents of the pro-thrombinase and extrinsic tenase complex. Expected inhibition of both tenases and the pro-thrombinase activity by binding of VaaSPH-1 to constituents of these complexes was confirmed in vitro (~Fig. 6 and ~Supplementary Fig. S4, available in the online version). VaaSPH-1 inhibited the activity of pro-thrombinase and extrinsic tenase in vitro even more efficiently than the activity of intrinsic tenase complex. This may be the consequence of a very complicated in vitro experimental setup in the case of the intrinsic tenase complex interference assay, including in situ activation of FVIII with thrombin and subsequent inhibition of thrombin with an overload of hirudin, an SP inhibitor. Also, other authors reported that the results in this kind of experiment are highly dependent on concentration, activity and time of incubation of FVIIIa with other coagulation factors.⁶³ Substantially higher inhibition (for 25%) of the intrinsic tenase activity, when VaaSPH-1 was added before FIXa in the assay (~Fig. 6C), and the inability of VaaSPH-1 to inhibit hydrolysis of a small substrate by FIXa (~Fig. 6D), proposed FVIIIa as the main target of VaaSPH-1 in the intrinsic tenase complex. Conclusively, our experimental data suggest that the anticoagulant

activity of VaaSPH-1 is significantly due to its competitive binding to the FIXa-binding site on FVIIIa and consequent inhibition of the intrinsic tenase complex formation.

We decided to test this hypothesis by an *in silico* approach. To this end, we constructed a 3D model of VaaSPH-1. This was made by the crystal structure of AHV_TL-1,⁴⁶ a SV trypsin-like SP with the highest homology to VaaSPH-1. We compared the obtained VaaSPH-1 space model with the hc FIXa and hc FX. Evaluation resulted in RMSD values of 1.2 and 1.8 Å, respectively, for the first and the second pair of molecules. Such results confirmed the view that VaaSPH-1 interferes with the function of the intrinsic tenase complex principally by mimicking FIXa and not by displacing its substrate, FX—that is, it acts as an antagonist of FIXa binding to FVIIIa. In the next stage, we replaced the FIXa unit with either hc FIXa or VaaSPH-1 in the structure of the intrinsic tenase complex on the PL membrane as constructed by Venkateswarlu.²⁵ Structures were docked using the HADDOCK program to reach thermodynamically most optimal states (—Fig. 7A and B). In such state, Van der Waals, electrostatic and desolvation energies were calculated for each complex (—Fig. 7C). Confirming our hypothesis, the structure, in which VaaSPH-1 was replacing FIXa in the complex proved energetically as stable as the native intrinsic tenase complex. The complex harbouring just the SP domain of FIXa (hc FIXa) was considerably less stable, which was not a surprise, as FIXa forms contacts with FVIIIa, not only with its SP domain, but also with its GLA and EGF domains.²⁵ This denoted that the interaction between VaaSPH-1 and FVIIIa is tighter than the interaction between hc FIXa and FVIIIa, particularly at the expense of the electrostatic energy (—Fig. 7C).

Considering the FVIIIa interaction surface, the so-called 558-loop in its A2 domain is involved in the interaction with hc FIXa,⁶⁴ that is, with its 157-loop (174-loop in chymotrypsin numbering) and the preceding α -helix (—Fig. 7A and B). A contact region between hc FIXa and FVIIIa is expanded in the inset of —Fig. 7A, while the one between VaaSPH-1 and FVIIIa is expanded in the inset of —Fig. 7B. Closer views explain larger electrostatic stabilization of the VaaSPH-1/FVIIIa complex by one additional ionic and some hydrogen bond interactions in comparison to the hc FIXa/FVIIIa complex.

Disturbance in the intrinsic coagulation cascade is the main reason for blood hypercoagulability in veins,^{21,34} therefore inhibition of blood coagulation by obstruction of the FVIIIa-FIXa interaction has been perceived as an attractive possibility for the treatment and prevention of diseases caused by VTE.³⁴ Based on the notion that the main interaction between FIXa and FVIIIa to form a functional intrinsic tenase complex includes the 558-loop in FVIIIa, linear and cyclic peptides or peptidomimetics have been designed and tested for their anticoagulant activity.^{34,65} Although these studies confirmed the FVIIIa 558-loop (Ser558-Gln565) as a promising model structure to design low molecular mass anticoagulants, there was no follow-up, probably due to too low efficacy of the tested compounds. Epidemiologic studies showed that the highly active FVIIIa

represents a significantly higher risk for thrombosis,⁶⁶ rendering this particular blood factor a drug target of a high relevance. VaaSPH-1 with its much tighter interaction with FVIIIa than hc FIXa offers therefore an excellent opportunity to employ a similar approach as described by Anastasopoulos et al.^{34,65} however, to design innovative FVIIIa-directed anticoagulants by taking the VaaSPH-1 structural elements, the 157-loop and the preceding α -helix, as a template. Importantly, effective reversal agents, recombinant FVIII and FIX,^{63,67} are already available for potential VaaSPH-1-based anticoagulant drugs.

In summary, from the venom of the European most venomous snake, we isolated and characterized a protein, VaaSPH-1, which possesses some unique traits—it is a very potent anticoagulant, although structurally an SP, it does not possess the enzymatic activity, it interferes with the GLA-domain-containing blood coagulation factors and with the SP-binding domain of the cofactors, FVa and FVIIIa. Focused on its interference with the intrinsic pathway of blood coagulation, we constructed a 3D model of its complex with FVIIIa that will advance a design of original low molecular mass FVIIIa-targeted anticoagulant substances based on the detailed analysis at the atomic level of the interaction surface between VaaSPH-1 and FVIIIa.

What is known about this topic?

- Components of the intrinsic blood coagulation pathway, among them FVIIIa, have been recognized as suitable therapeutic targets to treat venous thromboembolism.
- Complications, such as excessive bleeding, are frequent upon therapy with contemporary anticoagulants.
- Snake venoms are a rich source of proteins that affect blood coagulation.

What does this paper add?

- Comprehensive description of an anticoagulant snake venom protein, VaaSPH-1, that inhibits intrinsic tenase by binding to FVIIIa.
- Identification of the first FIXa antagonist of a serine protease structure.
- Construction of the three-dimensional model of VaaSPH-1-FVIIIa complex, for rational design of innovative FVIIIa-directed intrinsic tenase inhibitors of therapeutic relevance.

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Conflict of Interest
None.

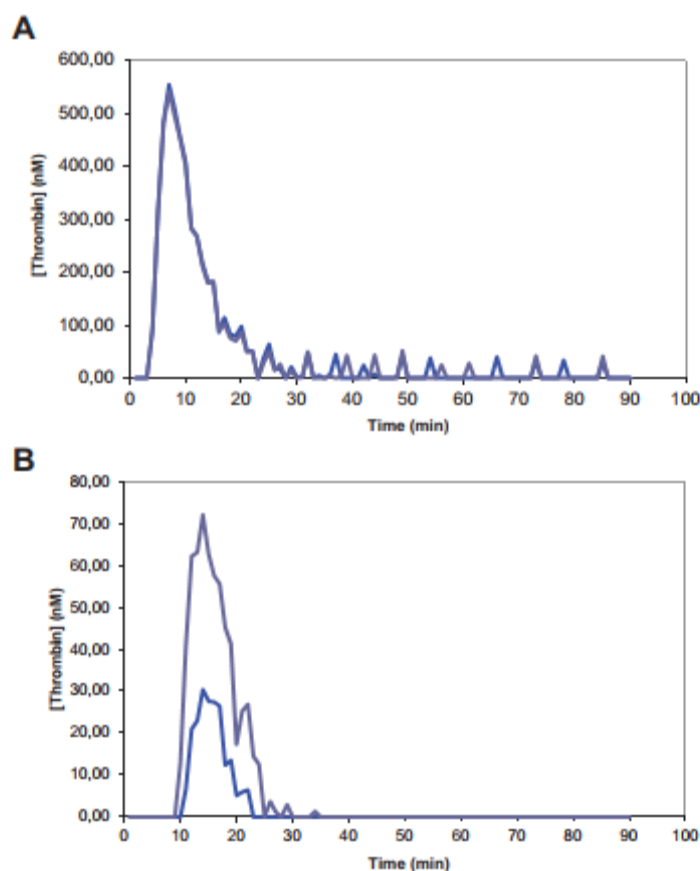
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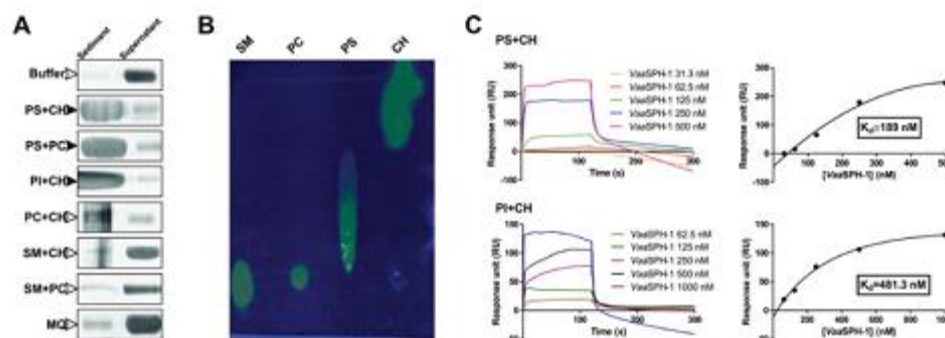
Supplementary Fig. S1. VaoSPH-1 almost completely abolishes formation of thrombin in blood. Thrombin generation assay was used to assess the capacity of VaoSPH-1 to suppress the generation of thrombin. Human plasma was incubated with 0.77 μ M VaoSPH-1 or Milli-Q water (control) for 2 minutes at 37°C. By addition of less than 5 pM TF, we activated both the intrinsic and the extrinsic pathway of blood coagulation. Thrombin activity was measured fluorimetrically using Technothrombin TGA substrate and Victor X2 multilabel plate reader (Perkin Elmer, Finland). Fluorescence was induced at 355 nm and recorded at 460 nm. From the intensity of the emitted fluorescence, concentration of thrombin was calculated using Technoclone software (www.technoclone.com). The area under the concentration curve corresponds to the total thrombin potential. Each experiment was performed twice (blue and violet trace).

Binding of VaoSPH-1 to lipids

Binding of VaoSPH-1 to Lipids by Sedimentation Assay

Phospholipid multilamellar vesicles (MLVs) were prepared in a 10-mL vacuum flask. Phospholipids (PLs) in the final concentration of 10 mg/mL were dissolved in chloroform. Solutions of PLs were pipetted in the vacuum flask in a way that the final mass of MLVs in 1 mL was 5 mg. MLVs consisted of sphingomyelin (SM) and phosphatidylcholine (PC); SM

and cholesterol (CH); phosphatidylinositol (PI) and CH; PC and phosphatidylserine (PS); PC and CH; and PS and CH in all cases in the molar ratio 1:1. Applying vacuum, chloroform was evaporated from the flask and a lipid film was formed on the bottom of the flask. This was dried for 2 additional hours. MLVs were formed from lipid films by addition of 1 mL of buffer C (20 mM Tris, 5 mM EDTA, 140 mM NaCl, pH 7) and intensive vortexing in the presence of acid-washed glass beads. Note that 20 μ L of MLV suspension was added to



Supplementary Fig. S2 VaaSPH-1 binds to negatively charged PLs. The binding of VaaSPH-1 to PLs and its charge selectivity was explored using three different experimental approaches, PL vesicle sedimentation assay (A), thin-layer chromatography (TLC) – blotting (B) and surface plasmon resonance (SPR) (C). Sedimentation assay showed whether VaaSPH-1 binds to multilamellar vesicles (MLV) or not. MLV varied in their PL composition, consisting of sphingomyelin (SM) + phosphatidylcholine (PC); SM + cholesterol (CH); phosphatidylinositol (PI) + CH; PC + phosphatidylserine (PS); PC + CH and PS + CH in the molar ratio 1:1. VaaSPH-1 was present in sedimented MLVs of PS + PC, PS + CH and PI + CH composition. If mixed with the MLV of other composition, VaaSPH-1 was found in the supernatant after the centrifugation (A). Individual PLs were chromatographed on a thin layer of silica gel. From the developed TLC plate, PLs were transferred/blotted onto the PVDF membrane, and analysed for binding of VaaSPH-1 using detection with anti-Vaa serum. By overlaying immunostained PVDF membrane and the silica gel plate, we concluded that VaaSPH-1 bound to negatively charged PS and PI, but not with SM, PC or CH (B). The selective binding of VaaSPH-1 to membranes containing the negatively charged PS or PI was confirmed using SPR (C). VaaSPH-1 interacted only with SUV of PS + CH and PI + CH, while no binding was observed with SUV of other compositions (PC + PS, SM + CH, SM + PC, PC + CH). Using this technology, we also determined the dissociation constant (K_D) for the interaction of VaaSPH-1 with SUV consisting of PS + CH and PI + CH in the molar ratio 1:1. In the first instance, K_D was 189 nM, while in the second 481.3 nM (C).

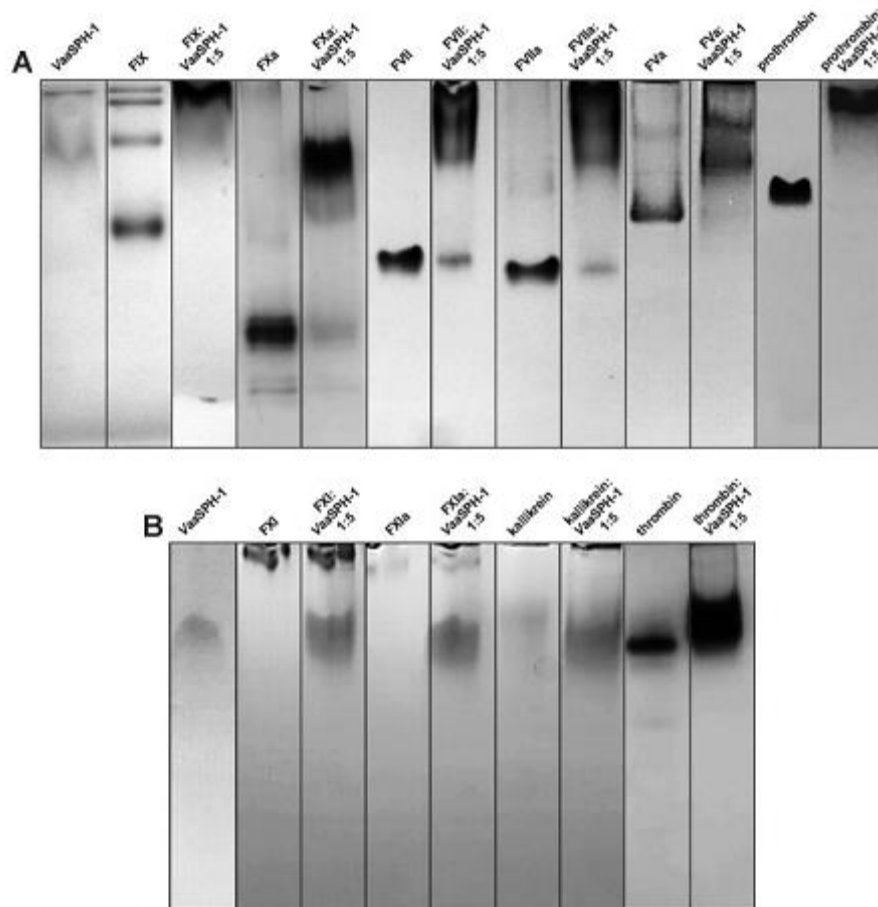
20 μ L of 0.5 mg/mL VaaSPH-1 in buffer C and incubated at the RT with shaking for 30 minutes. In negative controls, the same amount of VaaSPH-1 was added in three-fold Milli-Q water and buffer C. Following incubation, the samples were centrifuged at 60,000 g for 60 minutes at 4°C. Supernatants were transferred to fresh test tubes, supplemented with 12.5 μ L of 100% trichloroacetic acid and incubated for 10 min on ice. Then the test tubes were centrifuged at 16,100 g for 12 minutes at 4°C. In each test tube, supernatant was removed and 300 μ L of ice-cold acetone was added to the sediment. Samples were centrifuged at 16,100 g for 5 minutes at 4°C, followed by removal of supernatant and addition of ice-cold acetone. After the centrifugation at 16,100 g for 5 minutes at 4°C, supernatants were removed and sediments air-dried. Sediment and supernatant were analysed with 12.5% SDS-PAGE, under reducing conditions. Proteins were visualized by silver staining, as described in the 'Material and Methods' section.

Binding of VaaSPH-1 on the PVDF Membrane-Blotted Lipids

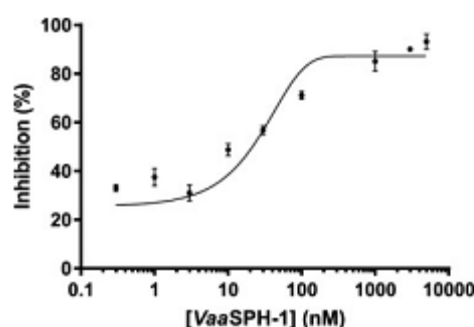
Thin-layer chromatography was performed on the heated aluminium silica gel plate (60 W, F254s: a fluorescent indicator coating silica gel, 20 $\times$ 20 cm, Merck, Germany). Note that 60 μ L of PLs (SM, PC, PS and CH) were applied. The plate was immersed in a solvent or a mobile phase, consisting of chloroform, methanol and ammonium in the

volume ratio 20:20:1, in chromatographic chamber, where the separation was accomplished. After the separation of PLs, the plate was dried to completely remove the solvent. PLs were transferred onto the PVDF membrane (Serva, Germany) by ironing (30 seconds at 180°C). The PVDF membrane was blocked for 2 hours at the RT in 5% (m/v) bovine serum albumin (BSA) (Merck, Germany) in TBS (50 mM Tris/HCl, pH 7.5 and 150 mM NaCl). Then the membrane was incubated for 2 hours with shaking at the RT in the blocking solution supplemented with 2 μ g/mL VaaSPH-1. After washing the membrane with TBS, it was incubated with the rabbit anti-Vaa serum, diluted 1:10,000, for 1 hour at the RT in TBS containing 5% (m/v) BSA and then with the horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgGs (diluted 1:10,000 in TBS with 5% (m/v) BSA) for 1 hour at the RT. PLs remaining on the silica plate were then detected by immersing the plate for 10 minutes in the mixture of 10 mL of methanol containing 30 mg of 4-chloro-1-naftol and 50 mL of 137 mM NaCl, 0.72 mM Na_2HPO_4 , 25 mM Tris and 60 μ L of H_2O_2 in water. Then the plate was sprayed with primulin solution (5 mg primulin in 100 mL 80% (v/v) acetone in water) to visualize PL spots. Processed PVDF membrane and silica plate were captured with G:BOX machine (Syngene, USA) and electronically overlaid to identify the nature of PL to which VaaSPH-1 bound.

VooSPH-1: The First SP-Like FIXa Antagonist Latinović et al.



Supplementary Fig. S3 Interaction of VooSPH-1 with different coagulation factors by native PAGE. Each blood coagulation factor was mixed with VooSPH-1 in the indicated molar ratio, and incubated at 37°C and pH 6.8 for 5 minutes. Samples were analysed by PAGE on continuous 10% (m/v) polyacrylamide gels under non-denaturing conditions to analyse whether VooSPH-1 and a coagulation factor form the complex or not. Lanes in which only VooSPH-1 or a coagulation factor was electrophoresed serve to define positions of non-complexed proteins. Appearance of an extra protein band upon analysis of the VooSPH-1 and a coagulation factor mixture indicated the complexing of the two molecules. The gels were stained with PageBlue. In panel (A), complexing of VooSPH-1 with FIX, FXa, FVII, FVIIa, FVa and prothrombin are demonstrated. (B) VooSPH-1 did not associate with FXI, FXIa, kallikrein and, consistently with the negligible influence of VooSPH-1 on TT, also thrombin.



Supplementary Fig. S4 VaaSPH-1 inhibits also extrinsic tenase activity. FVIIIa in buffer E and Innovin (contains recombinant human TF, PLs and Ca^{2+} in HEPES buffer; Dade Behring, Marburg, Germany) were added to each well of the 96-well microtiter plate and incubated for 15 minutes at 37°C for extrinsic tenase complex to form. To the complex, the increasing concentrations of VaaSPH-1 were added, incubated for 15 minutes, and then the substrate, FX, was added for 15 minutes. The reaction was stopped by adding the buffer E supplemented with 50 mM EDTA. Activity of the extrinsic tenase-generated FXa was determined using a chromogenic substrate S-2765 and A_{405} recording. The activity of FXa formed in the absence of VaaSPH-1 was considered 100% (0% inhibition). Percentages of inhibition by different concentrations of VaaSPH-1 were calculated using the formula: % inhibition = $\frac{(\text{reaction velocity in the absence of [VaaSPH-1]} - \text{reaction velocity in the presence of [VaaSPH-1]})}{\text{reaction velocity in the absence of [VaaSPH-1]}} \times 100$. All experiments were performed in duplicates and results are displayed as mean $\pm$ standard deviation (SD).

Binding of VaaSPH-1 on Lipids by Surface Plasmon Resonance

Small unilamellar vesicles (SUVs) were obtained from MLVs with 30 minutes of sonication (pulse on and off for 15 seconds, amplitude 50%) and 45 minutes of heating at 40°C, followed by centrifugation at 6,000 rpm for 5 minutes. SUVs were prepared in buffer D (20 mM Tris, 140 mM NaCl, 5 mM CaCl_2 , pH 7.4). This was also the buffer to perform experiments on the Biacore T-100 apparatus (GE Healthcare BioSciences AB, Sweden). SUVs were attached on the Biacore T-100 L1 chip in a flow cell within 10 minutes at a flow rate 2 $\mu\text{L}/\text{min}$. To prevent the non-specific binding, the chip was subsequently exposed for 2 minutes to 0.1 mg/mL BSA at a flow rate of 10 $\mu\text{L}/\text{min}$. Experiments with VaaSPH-1 in the flow cell 1 have been executed by applying the protein to the chip for 2 minutes at 10 $\mu\text{L}/\text{min}$ at different concentrations, from 31.25 nM to 500 nM in case of SUVs containing PS and CH (molar ration 1:1) and 62.5 nM to 1 μM in case of SUVs containing PI and CH (molar ration 1:1). Regeneration of the L1 chip was accomplished in the flow cell using 40 mM octyl β -D-glucopyranoside until the baseline was reached. The chip in the reference flow cell 2 was used to record the background signal. It was treated in an identical manner as the chip in the flow cell 1, just without the addition of VaaSPH-1. The dissociation constant K_d was calculated using Biacore T-100 Evaluation Software.

3.6 Znanstvena publikacija 6: Prokoagulantna serinska proteaza iz modrasovega strupa verjetno aktivira koagulacijska faktorja V in X

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SP iz KS so že izkazale visok terapevtski potencial v antikoagulantni terapiji (na primer Viprinex® in Defibrase®). Ob izolaciji *VaaSPH-1* smo s kationske kolone eluirali tudi aktivne SP, med katerimi je izstopala molekula, ki je v preliminarnih poskusih izrazito skrajšala čas koagulacije krvi. Tudi molekule, ki delujejo prokoagulantno, so zanimive terapevtsko in diagnostično, zato smo jih detajlno raziskali.

Molekula ni cepila α -, β - ali γ -verige fibrinogena, prav tako ni imela vpliva na agregacijo trombocitov. Sledilo je sistematično iskanje substrata izolirane SP v koagulacijski kaskadi, ki je privedlo do ugotovitve, da cepi tako FV kot FX. Z N-terminalnim sekveniranjem nastalih fragmentov smo določili mesta cepitve in pokazali, da encim verjetno cepi FV na istem mestu kot njegov naravni aktivator FXa, v FX pa cepi isto peptidno vez kot naravna aktivatorja, FVIIa in FIXa. Torej izolirana SP pretvori FV in FX v ustrezni aktivni obliki, FVa kot FXa, ki se združita v protrombinazni kompleks, ključnega pomena za koagulacijo krvi, saj pretvarja protrombin v trombin. Ker prokoagulantno delovanje SP, izolirane iz modrasovega strupa temelji, na aktivaciji FV in FX, smo molekulo poimenovali *VaaSP-VX*.

VaaSP-VX je ~34 kDa velik glikoprotein s heterogeno izoelektrično točko v območju pH 6–10. Aminokislinsko zaporedje smo določevali z N-terminalnim sekveniranjem po Edmanu in tandemsko MS ter določili 40 % celotnega zaporedja zrele oblike proteina. V cDNA knjižnici modrasove strupne žleze nismo našli proteina, ki bi bil popolnoma identičen *VaaSP-VX*, smo pa našli zapis za molekulo *VaaSP-6* [15], ki je 97 % identična molekuli *VaaSP-VX* na znanih delih strukture. Isto stopnjo identičnosti z znanimi deli *VaaSP-VX* deli tudi nikobin iz strupa *Vipera nikolskii* [128].

V primerjavi s trombinom, ki je tudi naravni aktivator FV in FX, SP iz KS nimajo dveh strukturnih zank, zato serpini, endogeni inhibitorji v krvi, kot je recimo in, ne morejo inhibirati njihovega delovanja. Iz tega razloga je *VaaSP-VX* primerna spojina vodnica za pripravo tako diagnostičnega orodja za določanje časa strjevanja krvi kot tudi novega prokoagulantnega zdravila, s katerim bi lahko zdravili hemofilike.

Moj prispevek k temu znanstvenemu delu je bilo celotno eksperimentalno delo, z izjemo sekvenčnih analiz, ter priprava prvega osnutka članka in priprava slik.



Article

The Procoagulant Snake Venom Serine Protease Potentially Having a Dual, Blood Coagulation Factor V and X-Activating Activity

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Abstract: A procoagulant snake venom serine protease was isolated from the venom of the nose-horned viper (*Vipera ammodytes ammodytes*). This 34 kDa glycoprotein, termed VaaSP-VX, possesses five kDa N-linked carbohydrates. Amino acid sequencing showed VaaSP-VX to be a chymotrypsin-like serine protease. Structurally, it is highly homologous to VaaSP-6 from the same venom and to nikobin from the venom of *Vipera nikolskii*, neither of which have known functions. VaaSP-VX does not affect platelets. The specific proteolysis of blood coagulation factors X and V by VaaSP-VX suggests that its blood-coagulation-inducing effect is due to its ability to activate these two blood coagulation factors, which following activation, combine to form the prothrombinase complex. VaaSP-VX may thus represent the first example of a serine protease with such a dual activity, which makes it a highly suitable candidate to replace diluted Russell's viper venom in lupus anticoagulant testing, thus achieving greater reliability of the analysis. As a blood-coagulation-promoting substance that is resistant to serpin inhibition, VaaSP-VX is also interesting from the therapeutic point of view for treating patients suffering from hemophilia.

Keywords: FV activator; FX activator; procoagulant; snake venom; serine protease

Key Contribution: We have characterized the first procoagulant snake venom serine protease that apparently activates both blood coagulation factors V and X. According to its structure, the protease is also resistant to serpin inhibition. Such features make this molecule highly medically relevant for diagnostic and therapeutic purposes.

1. Introduction

Snakes use their venom for defense and to immobilize and digest their prey. These functions are accomplished by enzymatic and/or non-enzymatic components of the venom and are usually characterized by high target specificity and effectiveness, thermal stability, and resistance to proteolysis [1]. The venoms of *Viperidae* snakes are rich in proteins that strongly affect the hemostatic

system by promoting or inhibiting platelet aggregation, blood coagulation, and fibrinolysis [2]. Members of the two families of snake venom enzymes, serine proteases (SVSPs) and metalloproteases (SVMPs), exert procoagulant activity by activating one or more coagulation factors in the blood coagulation cascade [3]. Depending on their principal target in the coagulation system, they are usually thrombin-like enzymes (TLEs), activators of prothrombin, factor V (FV), and FX. TLE and FV activators are exclusively SVSPs, while activators of FX and prothrombin can be either SVSPs or SVMPs [4].

SVSPs are abundantly present in viperid venoms, mostly as monomeric glycoproteins of 26 to 67 kDa. Their mass depends on the extent of either N- or O-glycosylation. SVSPs are classified within the clan PA as subclan S, family S1 (chymotrypsin), or subfamily A of the proteolytic enzymes [5]. Such proteases are characterized by a typical chymotrypsin fold and two six-stranded β -barrels. Their active sites lie in the cleft between the latter and include the canonical catalytic triad His-Asp-Ser [6]. Despite having a 50% to 80% shared identity of their amino acid sequences, SVSPs differ substantially in their substrate specificity, and consequently, in their pharmacological activity [7]. SVSPs that affect hemostasis express trypsin-like activity, hydrolyzing the BApNA (N α -benzoyl-L-arginine 4-nitroanilide) substrate. They are not inhibited by endogenous serine protease inhibitors (serpins) in the presence of heparin [8]. Consequently, SVSPs can be used in medical treatments and the diagnosis of blood coagulation irregularities [9]. The two best-known SVSP-based medical products are Viprinex® and Defibrase®. Viprinex® (also known as Ancrod) is a defibrinogenating agent derived from Malayan pit viper venom and has been investigated for the treatment of acute ischemic stroke [10]. Defibrase® is a therapeutic product based on a procoagulant SVSP called batroxobin from *Bothrops atrox* venom. It is used to decrease fibrinogen levels, leading to the inhibition of thrombogenesis and the prevention of thrombotic diseases. Batroxobin, sold as Reptilase®, is also used to determine the blood clotting time (Reptilase® time) [11].

The nose-horned viper (*Vipera ammodytes ammodytes* – Vaa), the most venomous snake in Europe, exhibits diverse hematotoxic and coagulopathic effects *in vivo* [12,13] and *in vitro* [14]. Vaa venom contains diverse types of enzymes, such as secreted phospholipases A₂ (sPLA₂s), SVMPs, L-amino acid oxidases (LAOs), and SVSPs. Although the latter are highly abundant in the venom, they have not been the most widely studied.

Four Vaa SVSPs have so far been purified and characterized: two kallikrein-like enzymes [15]; a fibrin(ogen)ase with an unconventional catalytic triad, VaSP1 [16]; and an anticoagulant, which is the enzymatically inactive SVSP homolog VaaSPH-1 [12]. Comprehensive transcriptomic and proteomic analysis of Vaa venom has revealed the existence of at least ten other SVSP molecules [17]. In this study, one of these SVSPs has been purified and characterized. VaaSP-VX, as we named this molecule, is a potent procoagulant. We have shown that its blood-coagulation-promoting effect is likely due to its FV- and FX-activating activities. The activation of the precursors of both components that form the prothrombinase complex by a single venom protease would be unique and would endow VaaSP-VX with promising medical potential.

2. Results

2.1. Isolation and Biochemical Characterization of VaaSP-VX

Vaa venom was size-fractionated into several protein peaks using gel filtration chromatography on a Sephacryl S-200 Superfine (Figure 1A). The B1 fraction, which contained proteases with molecular masses in the range of 30 to 40 kDa, was further separated using cation-exchange chromatography (Figure 1B). Eleven peaks were collected, dialyzed against buffer B, and tested for their effect on blood plasma coagulation. They exhibited either pro- or anti-coagulant activity (Figure 1C). We focused our attention on peak 10, which displayed the most pronounced procoagulant effect by shortening the prothrombin time assay (PT) and the activated partial thromboplastin time assay (aPTT). At the same time, this fraction did not influence the ADP- or collagen-induced aggregation, or ristocetin-induced agglutination of platelets (Figure 2A). As established using SDS-PAGE analysis and Edman sequencing, the fraction was homogenous, containing an SVSP with the N-terminal

amino acid sequence VIGGDECNINEHPFL. This procoagulant molecule was termed *VaaSP-VX*. From 1 g of the raw *Vaa* venom, we obtained 1 mg of pure *VaaSP-VX*, indicating a purification yield of 0.1%.

VaaSP-VX is an *N*-glycosylated protein of about 34 kDa (Figure 1D). When treated with peptide *N*-glycosidase F (PNGF), which cleaves off a glycan moiety from the *N*-glycosylated proteins, its molecular mass dropped by about 5 kDa. Typical of glycosylated proteins, *VaaSP-VX* was present in seven glycoforms, with isoelectric points (pIs) between 6 and 10 (not shown). The main glycoform was neutral, displaying a pI of approximately 7.

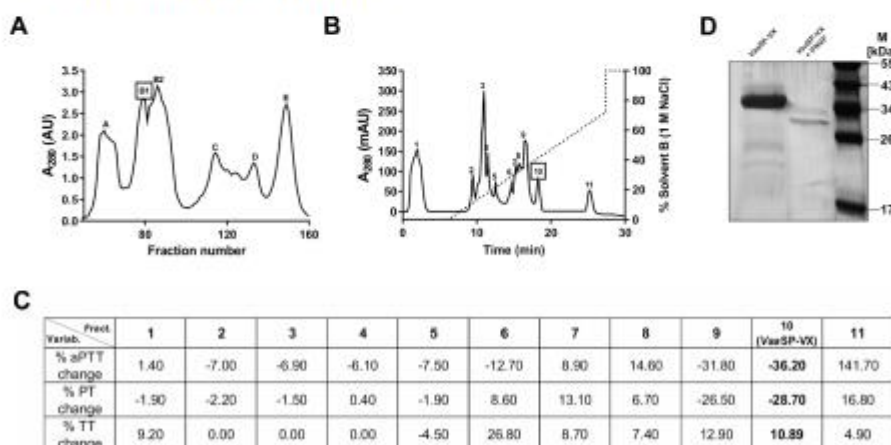


Figure 1. The purification and basic characterization of *VaaSP-VX*. (A) Size-exclusion chromatography of the raw *Vaa* venom was carried out on a Sephacryl S-200 superfine column. The elution of proteins was monitored at 280 nm (A_{280}). (B) Fraction B1 from the size-exclusion chromatography was sub-fractionated on a Mono S 5/50 GL ion-exchange fast protein liquid chromatography (FPLC) column. The retained proteins were eluted using a linear gradient of NaCl (dotted line) in 11 fractions. (C) Fractions were tested for their effect on blood coagulation. Three clinical blood coagulation assays were used: a prothrombin time assay (PT), an activated partial thromboplastin time assay (aPTT), and a thrombin time assay (TT). The table shows the effects of each fraction on aPTT, PT, and TT in terms of the percent change relative to the value obtained in the control experiment performed without the addition of a fraction. Fraction 10, which displayed a pronounced procoagulant effect, contained pure *VaaSP-VX* (values in bold). (D) Under reducing conditions, SDS-PAGE electrophoresis revealed *VaaSP-VX* to be a monomeric protein of about 34 kDa. Incubation of *VaaSP-VX* with peptide *N*-glycosidase F (PNGF) at 37 °C overnight followed by SDS-PAGE analysis displayed a lower molecular mass of about 29 kDa. *VaaSP-VX* was thus found to be an *N*-glycosylated protein.

2.2. *VaaSP-VX* Did Not Act Like Thrombin

To explain the molecular background of the pronounced procoagulant effect of *VaaSP-VX*, we examined whether *VaaSP-VX* acts like thrombin, i.e., whether it can clot fibrinogen directly. The specific blood coagulation assay, the thrombin time assay (TT), which would confirm the thrombin-like activity, showed only a slightly extended value of 10.89 (± 5.02 %) in the presence of 1 μ M *VaaSP-VX* (Figure 1C). In accordance with this, *VaaSP-VX* also failed to convert fibrinogen into a stable fibrin clot (Figure 2B). These results are consistent with the low fibrinogenolytic activity of the enzyme. As demonstrated using the SDS-PAGE analysis (Figure 2C), it only partially cleaved the α chain of fibrinogen, while the other two fibrinogen chains remained intact. It may thus be concluded that the acceleration of blood coagulation by *VaaSP-VX* was due to its action on blood coagulation factors in the coagulation cascade upstream of thrombin.

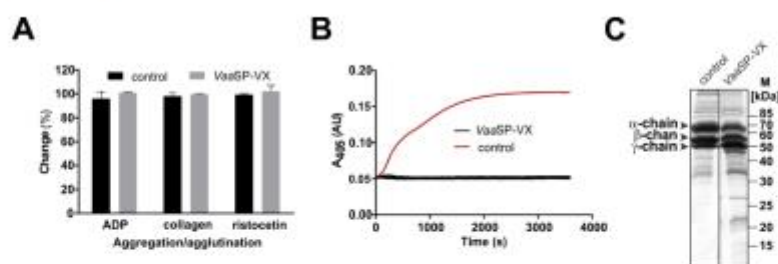


Figure 2. Effects of VaaSP-VX on platelets and fibrinogen. **(A)** Platelet-rich plasma was pre-incubated with 1 μ M VaaSP-VX at 37 $^{\circ}$ C. The agonists of platelet aggregation (ADP and collagen) or agglutination (ristocetin) were then added. Platelet aggregation/agglutination was followed by monitoring the optical density of the samples. Control values, obtained in the absence of the venom protein, were regarded as a 100% change of optical density. Evidently, VaaSP-VX did not affect the platelet function. **(B)** VaaSP-VX, in concentrations from 0.1 to 10 μ M, was added to human fibrinogen. Thrombin was used as a positive control. The formation of fibrin clots was tracked using continuous measurements of the absorbance at 405 nm (A_{405}). In the control experiment, thrombin induced a transformation of fibrinogen to fibrin, as indicated by the rise in A_{405} (red line). For all concentrations tested, VaaSP-VX did not stimulate clotting (black lines). **(C)** Human fibrinogen, consisting of α , β , and γ chains, was incubated with VaaSP-VX in a mass ratio of 100:1. In the control experiment, no VaaSP-VX was added. Following incubation at 37 $^{\circ}$ C for 60 min, the samples were analyzed on SDS-PAGE under reducing conditions and stained with PageBlue[®]. Only a slight degradation of the α -chain was apparent; therefore, the pronounced procoagulant effect of VaaSP-VX could not be due to its action on fibrinogen.

2.3. Activity of VaaSP-VX in Fresh Human Plasma

The addition of VaaSP-VX to fresh human plasma resulted in a dose-dependent shortening of aPTT and the re-calcification time compared with those of the control (Figure 3A,B). This indicated that VaaSP-VX activated one or more of the coagulation factors. Since PT, which reflects perturbations in the extrinsic pathway of blood coagulation, was shortened to almost the same extent as aPTT, which points to abnormalities in the intrinsic pathway of blood coagulation, we concluded that VaaSP-VX most probably interfered with blood coagulation somewhere in the cascade common to both pathways in the so-called common pathway of blood coagulation. This involved the prothrombinase complex, prothrombin, and fibrinogen. The prothrombinase complex is an assembly of activated FV and FX (FVa and FXa) on the negatively charged phospholipid membrane of platelets. Before proteolytic activation, FV and FX exist as soluble proteins in the blood. The prothrombinase complex activates prothrombin to thrombin, which then converts soluble fibrinogen to form an insoluble fibrin clot.

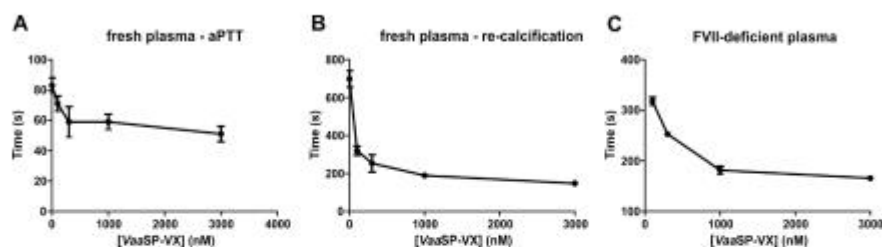


Figure 3. Clotting of fresh or coagulation factor-depleted plasmas in the presence of VaaSP-VX. (A) To fresh human plasma, VaaSP-VX was added at indicated concentrations and incubated at 37 °C for 10 min. The activated partial thromboplastin (aPTT) was then measured as described under Section 5: Materials and Methods. (B) To assess the plasma re-calcification time, the same procedure as in (A) was used, except the addition of the aPTT SA reagent was replaced by the addition of PBS. (C) To human plasma depleted in FVII, VaaSP-VX was added at the indicated concentrations. After 2 min of incubation at 37 °C, 25 mM CaCl₂ was added and the formation of fibrin clotting was observed by measuring the absorbance at 405 nm (*A*₄₀₅).

2.4. VaaSP-VX-Induced Clotting of Plasma Deficient in FVII

Commercial plasmas deficient in FVII or FIX only coagulate in the presence of Ca²⁺ ions if supplemented by phospholipids to enable the establishment of coagulation complexes, extrinsic or intrinsic tenase, and a prothrombinase complex, in which one or the other tenase complexes responsible for activation of FX is not present. Alternatively, such plasmas would coagulate only if VaaSP-VX were to activate FX directly or would act as FXa. Indeed, Ca²⁺-containing FVII-deficient plasma clotted in the presence of VaaSP-VX but remained unaffected if PBS alone was added (negative control). For example, 100 nM VaaSP-VX induced clotting of FVII-deficient plasma in 320 s. The effect was dose-dependent: the more VaaSP-VX that was added the quicker the clotting (Figure 3C). Such a result has led us to the conclusion that VaaSP-VX induced plasma clotting by stimulating the conversion of prothrombin to thrombin, either by activating FX to FXa or by acting in the same way as FXa.

2.5. VaaSP-VX Did Not Activate Prothrombin Directly

To test the hypothesis that VaaSP-VX induces a procoagulant effect by mimicking the action of FXa, we incubated prothrombin with VaaSP-VX. The formation of α -thrombin was probed using α -thrombin-specific fluorogenic substrate Boc-Val-Pro-Arg-AFC, whose hydrolysis was inspected by measuring the fluorescence at 500 nm. The substrate remained untouched; therefore, we concluded that VaaSP-VX was unable to activate prothrombin to α -thrombin. In agreement, VaaSP-VX was also not able to hydrolyze a chromogenic substrate (S-2222TM) that contains the FXa-cleavage site of prothrombin.

2.6. VaaSP-VX Activated FX and Likely Also FV

Next, we tested the hypothesis that VaaSP-VX induces the procoagulant effect by promoting the formation (and thus the activity) of the prothrombinase complex, i.e., by the activation of FX or/and FV. As is evident from Figure 4, VaaSP-VX was able to cleave both FV and FX. To verify whether the observed cleavages were converting these factors to their activated forms, we blotted the fragments from SDS-PAGE gels to polyvinylidene difluoride (PVDF) membranes and sequenced them.

VaaSP-VX initially cleaved bovine FV (in the first hour of incubation) into three main fragments with apparent molecular masses of 38, 55, and about 250 kDa (Figure 4A). The N-terminal sequence SLHLD of the 38 kDa fragment corresponded to part of the A2 domain of the bovine FVa. This fragment was excised from FV by two cleavages, after Arg³⁴⁸ within the A2 domain and at the N-terminus of the B domain (Figure 4C). The 55 kDa fragment, with an N-terminal sequence of AKLRQFY, corresponding to the FV domain A1, cleaved off FV at Arg³⁴⁸ (Figure 4C). The largest

fragment represented the rest of the FV molecule. It consisted of domains B, A3, C1, and C2 (Figure 4A,C). The fragment at about 50 kDa (Figure 4A) accumulated over time, with the N-terminal sequence ASSEV corresponding to the N-terminus of the light chain (LC) of FVa. This was a result of the sub-cleavage of the 250 kDa fragment after Arg¹⁷⁵³ within the A3 domain. *VaaSP-VX* thus cleaved bovine FV at the C-terminal end of Arg³⁴⁸ and Arg¹⁷⁵³, i.e., at the same sites as its natural activator FXa [18–20].

The result of the incubation of human FX with *VaaSP-VX* were two main protein fragments, one at 36 kDa and the other at 30 kDa (Figure 4B). The band at 42 kDa corresponded to the heavy chain (HC) of FX, while the band at 19 kDa corresponded to its LC, as we confirmed using Edman sequencing. We assumed that the band at 36 kDa was the FXa HC, the product of the removal of part the activation peptide (AP) from the N-terminus of the FX HC. The FXa HC was then further trimmed by *VaaSP-VX* somewhere at its C-terminal side to give the product FXa HC', which was 30 kDa in mass. The FXa HC' had the N-terminal sequence IVGGQ, which was the sequence of the FXa HC resulting from the cleavage of the FX HC after Arg¹⁹⁴ by physiological activators of FX, FVIIa, and FIXa (Figure 4D). Consistently, as FVIIa, *VaaSP-VX* hydrolyzed S-2288TM. However, the chromogenic substrates of FIXa and FXa were not cleaved by *VaaSP-VX*, offering us the possibility to test its FIX- and FX-activating abilities. Pre-incubation of FX with *VaaSP-VX* resulted in the hydrolysis of S-2222TM, which is the specific substrate of FXa. *VaaSP-VX* thus converted human FX into its active form, confirming the conclusion suggested by the SDS-PAGE analysis.

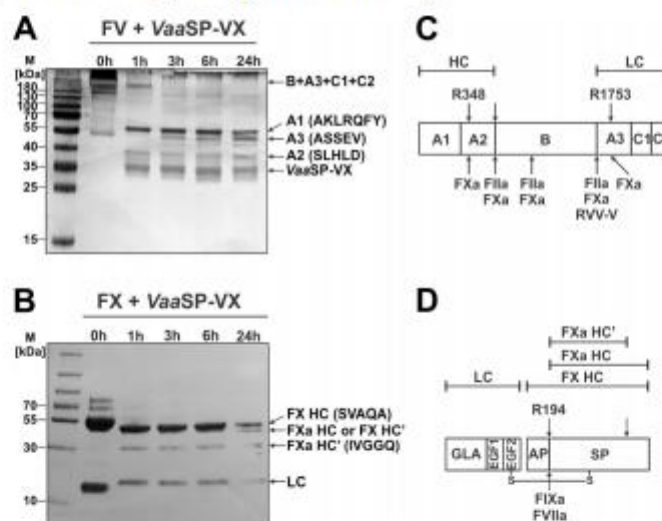


Figure 4. Hydrolysis of FV and FX by *VaaSP-VX*. Bovine FV (**A**), (or human FX (**B**)), was incubated with or without *VaaSP-VX*. An aliquot of the respective reaction mixture (10 μ L) was taken immediately (0 h) and then after 1, 3, 6, and 24 h of incubation at 37 $^{\circ}$ C; it was then analyzed on SDS-PAGE under reducing conditions. The gels were stained with PageBlue[®] to visualize the protein bands. As products of degradation by *VaaSP-VX*, the protein bands of FV or FX (arrows) were electroblotted to the PVDF membrane and N-terminally sequenced to define the cleavage positions. *VaaSP-VX* activated FV into FVa and FX into FXa. Schematic presentations of the domain structures of FV (**C**) and FX (**D**), with the indicated sites of activation by the following physiological activators: FXa, thrombin (FIIa), FVIIa, and FIXa, and RVV-V (below), and *VaaSP-VX* (above). Abbreviations: A1, A2, A3, B, C1, and C2: structural domains of FV; AP: activation peptide; EGF, GLA, and SP: epidermal growth factor-like, γ -carboxyglutamic acid-rich, and serine protease domain, respectively; LC, HC, and HC': light, heavy, and C-terminally truncated heavy chain, respectively.

2.7. Amino Acid Sequence of *VaaSP-VX*

Proteolysis of the purified *VaaSP-VX* and MS/MS analysis of the resulting peptides led to the establishment of 40% of the amino acid sequence (Figure 5). Using this sequence, we screened the non-redundant National Center for Biotechnology Information (NCBI) database and the in house *Vaa* venom gland cDNA sequence database. No complete match was found; therefore, we continued screening the cDNA library, using a PCR primer based on the N-terminal sequence of *VaaSP-VX*. As the search was not successful, a less-specific PCR primer was used directed toward cDNAs of all currently known SVSPs in the *Vaa* venom gland cDNA library [17]. In this way, a cDNA (MG958495), encoding *VaaSP-6*, which is a protein almost identical to *VaaSP-VX* in the known parts of its structure, was isolated (Figure 5). In the aligned regions, *VaaSP-VX* and the predicted mature form of *VaaSP-6* differ in only 3 of the 100 amino acid residues: Arg²², Ala⁴⁶, and Glu⁹² in *VaaSP-VX* are replaced by Thr, His, and Asn in *VaaSP-6*. *VaaSP-VX* and *VaaSP-6* may thus be considered to be isoforms. Searching in the non-redundant NCBI database revealed another SVSP with a high sequence similarity to that of *VaaSP-VX*, namely nikobin, from the venom of *Vipera nikolskii* [21]. The predicted amino acid sequence of the mature form of nikobin shared 97.42% identity with *VaaSP-6* and 97.00% with the sequenced parts of *VaaSP-VX* (Figure 5). Three potential N-glycosylation sites were conserved in *VaaSP-6* and nikobin (Asn¹¹⁰, Asn¹¹¹, and Asn²³⁹). Being located within sequences not covered by our MS/MS results, the existence of these sites was not confirmed in *VaaSP-VX*. *VaaSP-6* potentially contained an extra N-glycosylation site at Asn⁹², which was otherwise absent in *VaaSP-VX* and nikobin due to the Asn/Glu substitution.

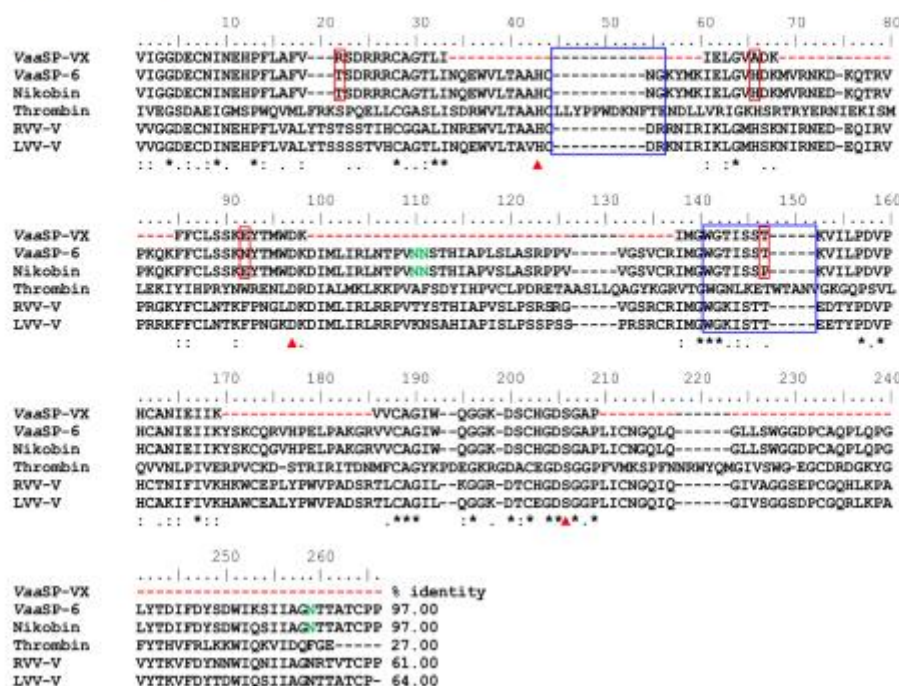


Figure 5. Partial amino acid sequence of VaaSP-VX and its comparison with sequences of some relevant serine proteases (SPs). VaaSP-VX was sequenced using a combination of Edman degradation and tandem MS. Based on its established partial structure, VaaSP-VX was recognized as an isoform of VaaSP-6, whose complete cDNA sequence was determined and deposited at the National Center for Biotechnology Information (NCBI, MG958495). Nikobin, an SP isolated from *Vipera nikolskii* venom, is a protein with the same extent of structural identity to the sequenced parts of VaaSP-VX as VaaSP-6 (97.0%). Amino acid residues, where differences between VaaSP-VX and VaaSP-6 or nikobin occurred, are in red boxes. Red arrows at the bottom of the aligned sequences point to three amino acid residues that constituted the catalytic triad in SPs (His57, Asp102, and Ser195, according to chymotrypsin numbering). The undetermined parts of the VaaSP-VX sequence are denoted by red dashes, while predicted gaps are denoted by black ones. Amino acid residues that may be N-glycosylated in VaaSP-VX (if present) are printed in green. Other SPs that were aligned are human thrombin, which is the main physiological activator of FV, and two snake venom FV activators, namely RVV-V from *Daboia siamensis* and LVV-V from *Macrovipera lebetina*. Except for thrombin, the two loops, 44-loop and 148-loop (in blue squares), are absent in all presented FV activators from snake venoms. This was established as the reason why snake venom SPs were resistant to inhibition by antithrombin. Below the sequences, an asterisk (*) designates a position that is strictly conserved in all aligned sequences, a colon (:) denotes a position in which only conservative substitutions were detected, and a dot (.) denotes a position harboring semi-conservative substitutions. On all other sites, the substitutions were non-conservative. The percentages of identity between the sequence of VaaSP-VX and the corresponding sequences in VaaSP-6, nikobin, thrombin, RVV-V, and LVV-V are presented.

3. Discussion

Vaa venom is a rich source of proteins that act on the hemostatic system [14]. In the present study, we purified and characterized a procoagulant SP from this venom and named it VaaSP-VX.

VaaSP-VX was isolated in a two-step chromatographic procedure, in which gel filtration was followed by cation-exchange chromatography (Figure 1). VaaSP-VX was found in the fraction, which

substantially shortened aPTT and PT without interfering with the platelet function (Figures 1C and 2A).

VaaSP-VX was found to be a single-chain N-glycosylated protein with an apparent molecular mass of 34 kDa. The mass of the glycan moiety was about 5 kDa (Figure 1D), representing approximately 15% of the total mass of the molecule. It was demonstrated that glycosylation affected the enzymatic activity and specificity of SVSPs by defining target recognition and by binding inhibitors [22–25]. Therefore, it may be expected that carbohydrates in VaaSP-VX have similar functions.

The partial amino acid sequence of VaaSP-VX was determined using Edman and MS/MS sequencing. The sequenced part of VaaSP-VX, which covered about 40% of the predicted mature protein, exhibited a high sequence identity with another SVSP from the *Vaa* venom, namely VaaSP-6 (MG958495), with unknown functions. These two proteins differed in the sequenced parts by only three amino acid residues, and therefore, could be regarded as isoforms (Figure 5). This conclusion is supported by a 2D-PAGE-based proteomic study of the *Vaa* venom [17], in which VaaSP-6 isoforms were identified in 21 spots positioned in the pI range from 6 to 9. Moreover, in 3 of these 21 spots, a peptide ²EYTMWDKDIMLR¹⁸ was detected, which was identified as a part of nikobin, which is an SVSP from the closely related snake *V. berus nikolskii* (E5AJX2) (Figure 5), but may also have originated from VaaSP-VX. The first part of this peptide, ²EYTMWDK⁸, which was only identified in the case of VaaSP-VX, was the same in the two molecules, as was the sequence preceding it, namely ⁸FFCLSSK⁹. Nikobin and VaaSP-VX were therefore identical in the region ⁸FFCLSSKEYTMWDK¹⁸, while VaaSP-6 was distinguished in this part from the other two SPs by the unique ²E/N substitution. In the non-redundant NCBI database, nikobin was the most similar protein to VaaSP-VX and VaaSP-6, displaying a 97% sequence identity (Figure 5). However, some VaaSPs may differ just in their glycosylation patterns, representing glycoforms of identical amino acid sequences.

The comparable effects of VaaSP-VX on aPTT and PT (Figure 1C) and clotting of FVII-deficient plasma by VaaSP-VX (Figure 3C) suggested that the action of this venom protease was directed toward the common pathway of the blood coagulation, in which FXa, as a component of the prothrombinase complex, activates prothrombin to thrombin, which then cleaves fibrinogen to form a fibrin clot [26]. We have shown that VaaSP-VX did not have the same effect as thrombin because it could not directly induce the clotting of fibrinogen (Figure 2B). Accordingly, the TT was also not shortened in its presence (Figure 1C), but moreover, it was even slightly extended. Such an effect may be explained by the defibrinogenating effect of VaaSP-VX due to its partial cleavage of the α chain of fibrinogen (Figure 2C). Since we showed that VaaSP-VX also did not possess FXa-like activity, its effect on blood plasma must be associated with the activity of the prothrombinase complex assembled from the activated forms of FV and FX. This suggested that VaaSP-VX was involved in the activation of these blood-clotting factors. To test this hypothesis, we treated FV and FX with VaaSP-VX and analyzed the cleavage products.

FV circulates in the blood as a 330 kDa protein comprised of six domains, A1, A2, A3, B, C1, and C2. In the process of its physiological activation, it is cleaved by thrombin to release the B domain. A heterodimeric FV is formed, in which the A1 and A2 domains constitute the HC and A3 and the C1 and C2 domains the LC, respectively [27]. This form of FV is further trimmed by FXa or by thrombin (FIIa). FXa cleaves FV at Arg³⁴⁸ in the A2 domain and at Arg¹⁷⁵³ in the A3 domain, which produces its fully activated form (Figure 4C) [18–20]. Bovine FV was incubated with VaaSP-VX and the degradation products were N-terminally sequenced. In this way, we established that VaaSP-VX cleaved FV at the same positions as FXa did. The report that treatment of human FV with FXa induced the FVa activity, which is associated with the cleavage at Arg¹⁷⁶⁸ (this is Arg¹⁷⁵³ in bovine FV) [28], strongly suggests that VaaSP-VX could convert not only bovine but also human FV into its activated form (Figure 4A,C). FV has already been identified as a target of SVSPs. Two well-studied examples are the FV activators from the venoms of *Daboia* snakes, 26 kDa RVV-V from *D. siamensis* and 28.4 kDa LVV-V from *V. (M.) lebetinus* [29,30]. However, they activate human FV in a thrombin-like manner, cleaving it at Arg¹⁵⁴⁵ (which corresponds to Arg¹⁵³⁶ in bovine FV; Figure 4C) [31]. Both enzymes are single-chain glycoproteins. Regarding VaaSP-VX, RVV-V appears in several isoforms

[29]. Two of them, which have been completely sequenced, differ from each other only by six amino acid residues.

Mature FX is a vitamin-K-dependent SP zymogen, which circulates in the blood as a disulphide-linked two-chain molecule of 59 kDa [32]. It is activated by FIXa or FVIIa, which are enzymatic components of the intrinsic or extrinsic tenase complex. In both cases, FXa is produced by cleavage of the Arg¹⁹⁴–Ile¹⁹⁵ peptide bond in the HC of FX. As we demonstrated, VaaSP-VX cleaved FX in the same way as both physiological activators (Figure 4B,D), and thus likely converted it into its activated form. Such an assumption was then also confirmed by the results of experiments using a specific chromogenic substrate of FXa. A variety of FX activators have already been found in snake venoms [4]. While FX activators from *Viperidae* venoms are mainly SVMPs, FX-activating SVSPs have only been described so far in two *Elapidae* venoms [33,34]. Like VaaSP-VX, they are single-chain glycoproteins but with a much higher molecular mass (62 and 70 kDa). No structural data are available for any of them; therefore, the first structural data of FX-activating SVSP is provided in this paper. The procoagulant activity of VaaSP-VX was thus the consequence of the simultaneous activation of FV and FX in a physiological manner by this venom enzyme.

The activity of blood coagulation factors is tightly regulated by endogenous protein inhibitors (serpins), such as antithrombin [35]. The serpin interaction site in thrombin includes its 44-loop and 148-loop (Figure 5). As was experimentally demonstrated using RVV-V and LVV-V [31], the absence of these two loops renders SVSPs resistant to serpins. VaaSP-VX also lacks these two loops; therefore, it is expected not to be inhibited by serpins. As an activator of multiple blood coagulation factors that is resistant to inhibition by serpins, VaaSP-VX is of potential interest regarding the development of an innovative clot-promoting drug for external use in patients suffering from hemophilia or other bleeding conditions [36].

Additionally, the potential double-activating activity of VaaSP-VX offers an elegant solution to replacing the unreliable diluted Russell's viper venom (dRVV) in clinical testing of the lupus anticoagulant (LA) [37]. Two components of dRVV are important for this test: RVV-V, which is an SVSP that activates FV and RVV-X, which is an SVMP that activates FX. However, the problem with the dRVV reagent is its consistency since it is obtained from a natural source that is subject to considerable biological variations. Accordingly, the results of the LA test are frequently inconsistent. To improve the sensitivity and specificity of the test, the use of purified snake venom components for the activation of FV and FX has already been suggested [38]. An even better solution would be to use VaaSP-VX, which would simultaneously activate both FV and FX.

4. Conclusions

We have purified and characterized the procoagulant SVSP from the venom of the nose-horned viper. This molecule, VaaSP-VX, seems to be unique in terms of its ability to activate FV and FX at the same time. As suggested by its structure, it also escapes inhibition by serpins. Such traits make VaaSP-VX medically interesting for replacing dRVV in LA testing and for developing novel blood-clot-promoting drugs.

5. Materials and Methods

5.1. Purification of VaaSP-VX

Crude Vaa venom was provided by the Institute of Immunology, Zagreb, Croatia. Lyophilized venom was kept at −20 °C. Before the analysis, it was dissolved in 50 mM Tris, 2 mM CaCl₂, 300 mM NaCl, pH 7.0 (buffer A). Fifteen milliliters of the crude venom solution (i.e., 1 g of the venom) was applied to a Sephacryl S-200 Superfine column (100 cm × 4 cm) (GE Healthcare BioSciences AB, Uppsala, Sweden). Gel chromatography was performed at a constant flow rate of 33.6 mL/h. The concentration of proteins in the mobile phase was monitored by measuring their absorbance at 280 nm (A₂₈₀). The B1 fraction was dialyzed against 20 mM MES, 2 mM CaCl₂, pH 6.5 (buffer B), and further fractionated on a Mono S 5/50 GL column of the FPLC system (ÄKTA, GE Healthcare Life-Science, Uppsala, Sweden), equilibrated with the same buffer. The column-retained material was

eluted using a linear gradient of NaCl (0 to 1 M) in the buffer B. *VaaSP-VX* was eluted from the column, concentrated, and dialyzed against buffer B, at pH 7.5.

5.2. Polyacrylamide Gel Electrophoreses

One-dimensional polyacrylamide gel electrophoresis (PAGE) of the samples (as specified in the descriptions of figures showing the results) was performed under non-denaturing or denaturing conditions, i.e., in the absence and presence of the detergent sodium dodecyl sulphate (SDS-PAGE). For SDS-PAGE analysis, 12.5% (*m/v*) PA gels were used under non-reducing and reducing conditions. The proteins in gels were visualized via silver staining using the modified method of Morrissey, where protein fixation is accomplished by a mixture of water, ethanol (30% (*v/v*)), and acetic acid (10% (*v/v*)) [39]. The molecular mass standards were from Fermentas (Thermo Fisher Scientific, Vilnius, Lithuania).

Two-dimensional gel electrophoresis (2DE) of 9 µg of *VaaSP-VX* was performed as specified in Latinović et al. [39].

5.3. N-Terminal Amino Acid Sequencing and Mass Spectrometry

N-terminal amino acid sequencing of the isolated *VaaSP-VX* was performed using automated Edman degradation on a Procise 492A Sequencing System (Applied Biosystems, Foster City, CA USA).

For the mass spectrometry (MS) analysis, 2 µg of vacuum-dried *VaaSP-VX* was dissolved in 10 µL of 50 mM NH_4HCO_3 supplemented with 6 M urea. The protein was reduced via the addition of 0.25 µL of 100 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 and incubation at 37 °C for 1 h. The mixture was then diluted with 10 µL of 50 mM NH_4HCO_3 and alkylated with 0.8 µL of 250 mM iodoacetamide in 50 mM NH_4HCO_3 at room temperature (RT) for 45 min in the dark. Alkylation was stopped by adding 2.5 µL of DTT and then 50 mM NH_4HCO_3 to 90 µL. Fragmentation of the reduced and alkylated *VaaSP-VX* was accomplished by adding 0.1 µg of endoproteinase Lys-C and standing at 37 °C overnight. Fifty nanograms of trypsin in 10 µL of 50 mM NH_4HCO_3 was then added, and the mixture was left overnight for further fragmentation at 37 °C. Proteolysis was stopped via the addition of formic acid to the sample to a final concentration of 0.1% (*v/v*). The sample was desalted and concentrated in Vivapure C18 micro spin columns (Sartorius Stedim Biotech, Göttingen, Germany) according to the manufacturer's instructions, with formic acid replacing trifluoroacetic acid. The *VaaSP-VX* hydrolysate was analyzed on an ion trap mass spectrometer 1200 series HPLC-Chip-LC/MSD Trap XCT Ultra (Agilent Technologies, Waldbronn, Germany), as described in Leonardi et al. [40]. MS and tandem MS (MS/MS) spectra were searched against the *Vaa* venom gland cDNA transcripts library and the non-redundant NCBI (National Center for Biotechnology Information) database using the Spectrum Mill software (A.03.03.084, Agilent Technologies, Santa Clara, CA, USA).

5.4. Search for the mRNA Transcript of *VaaSP-VX*

The cDNA sequence of *VaaSP-VX* was searched for using the *Vaa* venom gland cDNA library of mRNA transcripts [41], and PCR primers designed using two different approaches. First, we designed the sense primer deduced from an amino acid stretch from the N-terminal region of *VaaSP-VX* and determined using Edman degradation (CNINEHPF; cacgaattcATGTAACATAAATGAACATCCTTTC, where the additional *EcoRI* restriction site is underlined). Second, we designed a sense primer with a broader specificity (CNINEHP; cacgaattcATGTAACATAAATGAACATCCTTT). The antisense primer was the same in both cases and was deduced from the end of the coding region of other known *VaaSP* mRNAs, including the stop codon and a part of the 5' UTR region (tataagcTTTTCAAAAGTTTTCACGGGG, with an additional *HindIII* site underlined and a stop codon doubly underlined). In an attempt to obtain more specific binding of primers to template DNA, we employed different PCR protocols by using Taq or

Q5 polymerase, changing the number of cycles and T_m , and lowering concentrations of primers and dNTPs.

5.5. N-Deglycosylation

Four micrograms of dry VaaSP-VX were dissolved in 10 μ L of 1% (*m/v*) SDS and incubated at 100 °C for 5 min. The solution was mixed with 34 μ L of 1.5% (*m/v*) CHAPS in 50 mM Na₂HPO₄, pH 7.5. A total of 3 U (3 μ L) of the peptide N-glycosidase F (PNGF, Roche, Mannheim, Germany) was added to half of this sample. To the other half, just water was added and served as the control. After overnight incubation at 37 °C, both samples were analyzed using SDS-PAGE on a 12.5% gel under reducing conditions. The gel was stained with colloidal silver, as described above.

5.6. Prothrombin Time, Activated Partial Thromboplastin Time, Thrombin Time, and Re-Calcification Time Measurements

To 45 μ L of normal human plasma (Diagnostica stago, Asnières-sur-Seine, France) in a cuvette, we added an aliquot of each fraction eluted from a Mono S column in 5 μ L (volume adjustment was accomplished using buffer B) to obtain the final concentration of protein in the assay of 1 μ M. In the control experiment, just 5 μ L of buffer B was added to human plasma. Samples were incubated for 1 min at 37 °C. Then, the prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) were measured using the BFT II Coagulation Analyzer (Siemens, Munich, Germany). The measurement of PT was initiated by adding 100 μ L of Thromborel S reagent (lyophilized human placental thromboplastin (≤ 60 g/L) and CaCl₂ (approx. 1.5 g/L); Siemens, Munich, Germany). The measurement of aPTT was initiated by adding 50 μ L of Pathromtin SL reagent (SiO₂ particles (1.2 g/L), plant phospholipids (0.25 g/L), NaCl, HEPES, pH 7.6; Siemens, Munich, Germany). After 2 min of incubation, 50 μ L of 25 mM CaCl₂ was added and the clotting time was recorded as the aPTT. The addition of 100 μ L of Thrombin reagent (source of α -thrombin; Siemens, Germany) to the sample induced the clotting of plasma in a time registered as the TT.

The dependence of aPTT on the concentration of VaaSP-VX was also measured in an alternative way. Fresh human plasma, aPTT soluble activator (SA) reagent (Helena Laboratories, Beaumont, TX USA), 25 mM CaCl₂ (Helena Laboratories, USA), and solutions of VaaSP-VX in phosphate-buffered saline (PBS: 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) were pre-warmed at 37 °C. Different amounts of VaaSP-VX were added to the plasma, which was incubated for 10 min at 37 °C. The aPTT SA reagent (0.1 mM Ellagic acid with a suspension of phospholipids extracted from dehydrated rabbit brain) was then added and the mixture was incubated for another 5 min. Finally, the clotting of plasma was initiated by the addition of CaCl₂. All solutions were always added in 30 μ L volumes. The formation of the clot was followed by measuring the absorbance at 405 nm (A_{405}) on an Infinite M200 microplate reader (Tecan, Männedorf, Switzerland).

The same procedure, except substituting aPTT SA reagent with PBS (i.e., the procedure without supplement of phospholipids), was used to assess the full plasma re-calcification time.

The blood was donated by healthy human volunteers according to permission no. 53/08/11 of the National Medical Ethics Committee of the Republic of Slovenia.

5.7. Platelet Aggregation/Agglutination Assays

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as described in Leonardi et al. [42]. To 250 μ L of PRP, 25 μ L of VaaSP-VX solution was added, resulting in a final concentration of 1 μ M VaaSP-VX; it was then pre-incubated for 5 min at 37 °C. In the control experiment, 25 μ L of water was added to 250 μ L of PRP. Twenty-five microliters of collagen solution (DiaMed, Cressier, Switzerland), giving a final concentration of 10 μ g/mL; ristocetin (Chrono-Log Corporation, Havertown, PA, USA), resulting in a final concentration of 1 mg/mL; or ADP (Chrono-Log Corporation, USA) at a final concentration of 10 μ M, were lastly added and the change in the optical density was monitored for 5 min using a Model 700 Whole Blood/Optical Lumi-Aggregometer (Chrono-Log Corporation, Havertown, PA, USA). The cuvette containing PRP corresponded to 0%

light transmission and the cuvette with PPP to 100% light transmission. Blood was donated by healthy human volunteers according to permission no. 53/08/11 of the National Medical Ethics Committee of the Republic of Slovenia.

5.8. FVIIa, FIXa, and FXa-like Activity Testing

To test whether VaaSP-VX acts in the same way as FVIIa, FIXa, or FXa, we used the following specific chromogenic substrates: S-2288TM (Diapharma, West Chester Township, OH, USA) for FVIIa, Spectrozyme[®] (American Diagnostics Inc., New York, NY, USA) for FIXa, and S-2765TM and S-2222TM (Diapharma, West Chester Township, OH, USA) for FXa. To 100 μ L VaaSP-VX (diluted with PBS to final concentrations of 1 μ M and 3 μ M), 50 μ L of Spectrozyme[®], S-2288TM, S-2765TM, or S-2222TM were added to final concentrations of 1 mM. Substrate hydrolysis was then examined on an Infinite M200 microplate reader (Tecan, Switzerland) by measuring A_{405} .

Additionally, to test the FXa-like activity of VaaSP-VX on prothrombin, we incubated 10 pmol of prothrombin with 20 pmol of VaaSP-VX in 20 μ L of a buffer consisting of 50 mM HEPES, 2 mM CaCl₂, and 150 mM NaCl (pH 7.4). After 10 min of incubation at room temperature, the reaction mixture was 10-fold diluted with the buffer and transferred to a microplate well. After supplementing the reaction mixture with 10 μ M α -thrombin-specific fluorogenic substrate Boc-Val-Pro-Arg-AFC (Peptide Institute Inc., Osaka, Japan), the hydrolysis of the substrate was assessed on an Infinite M1000 microplate reader (Tecan, Switzerland) by activating fluorescence at 380 nm and detecting it at 500 nm.

5.9. Testing of FIX and FX Activation

The activation of FIX and FX by VaaSP-VX was determined using the following specific chromogenic substrates: Spectrozyme[®] (American Diagnostics Inc., USA) for FIXa and S-2765TM (Diapharma, USA) for FXa. In a well of a 96-well plate, 50 μ L of 1 μ M VaaSP-VX was added to 50 μ L of 100 nM FIX, and then incubated for 15 min at 37 °C. Furthermore, to 50 μ L of 100 nM FX, 3 μ M or even 10 μ M VaaSP-VX was added before the incubation. In negative controls, VaaSP-VX was replaced with PBS. Activation of a particular blood coagulation factor by VaaSP-VX was detected using an Infinite M200 microplate reader (Tecan, Switzerland). The activation of FIX or FX was detected via an increase in A_{405} following the addition of 50 μ L of Spectrozyme[®] (final concentration 1 mM) or S-2765TM (final concentration 0.65 mM) to the reaction mixture, respectively.

5.10. Characterization of Proteolytic Specificity

The proteolytic activity of VaaSP-VX was characterized by testing its ability to hydrolyze and/or activate blood coagulation factors and cofactors.

The fibrinogenolytic activity of VaaSP-VX was examined as described in Sajevec et al. [43] using a human fibrinogen to VaaSP-VX mass ratio of 100:1. It was then tested to determine whether the cleavage of fibrinogen by VaaSP-VX resulted in the formation of a stable fibrin clot. To this end, 40 μ L of VaaSP-VX in PBS (0.1 to 10 μ M final concentration), or 40 μ L of thrombin (final concentration 1 μ M; Helena Laboratories, USA) in a positive control, was added to a well of the 96-well microtiter plate. Subsequently, 40 μ L of fibrinogen (final concentration 2 μ M; Sigma-Aldrich, St. Louis, MO, USA) was added to a well and the fibrin clot formation was monitored at 405 nm (A_{405}) using a microplate reader (Infinite M200, Tecan, Switzerland).

To examine the hydrolysis of FV and FX (Haematologic Technologies Inc., Essex Junction, VT, USA) by VaaSP-VX, we used more easily accessible bovine FV, which appears to be functionally identical to human FV [19] and human FX. Seven micrograms of each substrate was incubated with or without (control) 1 μ g of VaaSP-VX in 25 μ L of 20 mM Tris/HCl, 5 mM CaCl₂, 140 mM NaCl, pH 7.5 at 37 °C. A 10 μ L aliquot of the reaction mixture was withdrawn after 1, 3, 6, and 24 h, and analyzed using 12.5% (w/v) SDS-PAGE under reducing conditions. The gels were stained with PageBlue[®] (Thermo Fisher Scientific, Vilnius, Lithuania). Protein bands were electroblotted from the gel to the PVDF membrane in Towbin transfer buffer (4 mM Tris/HCl, pH 7.5, 12 mM NaCl, and 20%

(v/v) methanol) at 200 mA per gel for 90 min at room temperature. The PVDF membrane was immersed in 100% methanol for a few seconds, then moved to a 0.1% (m/v) Coomassie Blue R-250 (Thermo Fisher Scientific, Vilnius, Lithuania) + 1% (v/v) acetic acid + 40% (v/v) methanol solution for 1 min. The membrane was destained in 50% (v/v) methanol, then washed with water. Protein bands were excised and sequenced using automated Edman degradation on a Procise 492A Sequencing System (Applied Biosystems, Waltham, MA, USA).

5.11. Clotting of FVII-Deficient Plasma

Seventy-five microliters of commercial human plasma lacking FVII (HemosIL®, Lexington, MA, USA), 95 µL of 25 mM CaCl₂ (Siemens, Germany), and 20 µL VaaSP-VX in PBS to various final concentrations (refer to Figure 3C for concentrations) were individually pre-warmed to 37 °C. VaaSP-VX (or only PBS in the control experiment) was then added to the plasma and incubated for 2 min at 37 °C, followed by the addition of 25 mM CaCl₂ to induce clotting. Plasma clotting time was measured using the BFT II Coagulation Analyzer (Siemens, Germany).

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Poglavje 4

Zaključki

V doktorski disertaciji sem se osredotočila na iskanje in karakterizacijo molekul iz strupov modrasa in gada, dveh medicinsko najbolj zanimivih evropskih kač. Modras je najbolj strupena, medtem ko je gad najbolj razširjena kača na stari celini. KS oziroma toksini, prisotni v strupih, so že nekaj desetletij atraktiven predmet znanstvenih raziskav, ker izražajo izrazite in specifične vplive na človeško telo. Komponente viperidnih strupov, ki učinkujejo na sistem hemostaze, so potencialno primerne za razvoj novih terapevtskih učinkovin za zdravljenje motenj koagulacije krvi oziroma bi lahko bile uporabne za krvno diagnostiko. Posebno pozornost smo zato usmerili v iskanje strupnih komponent, ki izražajo bodisi močno pro- bodisi antikoagulantno aktivnost na človeško kri. Tri take molekule iz modrasovega strupa smo podrobno biokemijsko karakterizirali in jim določili tudi molekularni mehanizem delovanja, saj je to osnova za potencialno uporabo v medicini.

Prvo terapevtsko zanimivo molekulo smo izolirali iz modrasovega strupa, in sicer P-III MP VaF1. Le-ta je visoko specifična α -fibrinogenaza brez fibrinolitske aktivnosti. Cepi le eno vez (Lys⁴¹³-Leu⁴¹⁴) v C-terminalnem delu A α -verige fibrinogena in s tem prepreči nastanek stabilnih fibrinskih strdkov. VaF1 je nehemoragična MP in ne vpliva na agregacijo trombocitov ter je zato primerna spojina vodnica za razvoj nove terapevtske učinkovine za defibrinogenacijo, kar potrjuje našo drugo hipotezo.

V modrasovem strupu smo zasledili še dve komponenti z zanimivim diagnostičnim kot tudi terapevtskim potencialom. Članek v poglavju 3.5 govori o antikoagulantni molekuli, medtem ko je v sekciji 3.6 opisana molekula s prokoagulantnim delovanjem.

Antikoagulantna molekula VaaSPH-1 je enoverižni glikoprotein, strukturni homolog SP, ki ji v primarni strukturi manjkata dve aminokislini iz katalitične triade pri SP. Njegovo delovanje je zaviranje notranje poti koagulacije, kar potrjuje našo tretjo hipotezo. 3D model kompleksa FVIIIa–VaaSPH-1 nam je omogočil vpogled v stično površino med molekulama. Identificirali smo del strukture VaaSPH-1, ki je ključen za vezavo na FVIIIa, to sta t. i. zanka 157 ter α -heliks, ki sledi zanki. Identificirani del strukture lahko služi kot osnovno ogrodje za pripravo inovativnih peptidov oziroma peptidomimetikov, ki bodo specifično inhibirali vezavo FIXa na FVIIIa in tako preprečili tvorbo kompleksa ter strjevanje krvi. Kot učinkoviti antidoti pa lahko služijo rekombinantno pripravljeni FVIIIa oziroma FIXa [129, 130]. Glede na to, da so glavni vzrok za hiperkoagulabilnost v žilah motnje v notranji koagulacijski poti [131, 132, 133], je inhibicija interakcije med FVIIIa in FIXa ena od zanimivih in varnih možnosti zdravljenja in preventive bolezni, povzročene z vensko trombembolijo. S tem smo dodatno potrdili našo tretjo hipotezo, ki trdi, da je v modrasovem strupu prisoten primeren protein kot spojina vodnica za razvoj nove in varne terapevtske učinkovine za zdravljenje venske trombembolije.

Nasprotno od antikoagulantnega VaaSPH-1 smo v poglavju 3.6 predstavili molekulo, ki deluje prokoagulantno oziroma skrajša čas strjevanja krvi. Poimenovali smo jo VaaSP-VX. Za razliko od VaaSPH-1 je molekula encimsko aktivna. VaaSP-VX verjetno cepi FV in FX in to tako, da ju pretvori v aktivno obliko. S tem pospeši tvorbo protrombinaznega kompleksa ter posledično

aktivacijo trombina in nastanek fibrina. Hkrati pa je verjetno odporna na inhibicijo s serpini zaradi odsotnosti strukturnih zank, kamor se vežejo serpini [134]. *VaaSP-VX* je tako zelo primerna spojina vodnica za razvoj novega zdravila, ki spodbuja nastanek strdka.

Poleg terapevtskega ima *VaaSP-VX* tudi diagnostični potencial. Glede na to, da verjetno simultano aktivira FV in FX, lahko nadomesti razredčen strup Russellovega gada pri testiranju lupusnih antikoagulantov. Predlagano je bilo že, da se namesto razredčenega strupa Russellovega gada uporabita dva izolirana aktivatorja [135]. V primeru *VaaSP-VX* bi zajeli obe aktivnosti v eni molekuli, kar je optimalna rešitev.

Z identifikacijo proteinov VaF1, *VaaSPH-1* in *VaaSP-VX*, ki imajo tako terapevtski kot diagnostični potencial pri kardiovaskularnih boleznih, smo potrdili tudi del naše prve hipoteze, ki trdi, da komponente modrasovega strupa vplivajo na koagulacijo krvi ter da je modrasov strup ustrezen vir za izolacijo molekul z omenjeno aktivnostjo.

Prva hipoteza pravi tudi, da so v modrasovem strupu prisotne komponente z vplivom na srce. Iskanje kardiotoksina v modrasovem strupu smo predstavili v dveh znanstvenih publikacijah (poglavji 3.2 in 3.3). V prvi znanstveni publikaciji, opisani v poglavju 3.2, smo prišli do zaključka, da imajo največji kardiotoksični učinek na izolirano podganje srce komponente velikosti ~14 kDa, izolirane po gelski kromatografiji v frakciji C. Vplivajo na srce, tako da izrazito znižajo srčni utrip in koronarni pretok, zvišajo dLVP in hkrati znižajo sLVP in razviti tlak v levem ventriklu (devLVP), kar vse skupaj vodi v asistolični srčni zastoj. Na zelo podoben način je deloval celoten modrasov strup [136], zato smo sklepali, da se glavna(e) kardiotoksična(e) učinkovina(e) nahaja(jo) v frakciji C.

V poglavju 3.3 smo opisali nadaljnjo identifikacijo kardiotoksičnih komponent v frakciji C. Le-to smo v ta namen dodatno ločili na podfrakcije, v katerih smo identificirali Atx, AntL₁, AntL₂, AntL in CRISP. Kardiotoksičen vpliv na izolirano podganje srce so imele le podfrakcije, ki so vsebovale AtnL in CRISP. Po izpostavitvi podganjega srca čistemu AtnL je bil vzorec biokemijskih markerjev enak kot pri izpostavitvi celotnemu modrasovemu strupu, zato lahko sklepamo, da je le-ta glavna kardiotoksična komponenta v modrasovem strupu. AtnL je v podganjem srcu izzval enake učinke kot celoten modrasov strup, z eno razliko. Antiventrikularni blok se je v primeru AtnL izničil po koncu poskusa, medtem ko pri podfrakciji, kjer je bil prisoten še CRISP, temu ni bilo tako. Zato sklepamo, da so za to dejanje odgovorni CRISPi, za katere so že pokazali, da inhibirajo napetostno odvisne ionske kanalčke ali s cikličnimi nukleotidi kontrolirane ionske kanalčke [137, 138].

V znanstveni publikaciji, opisani v poglavju 3.1, smo s proteomsko analizo gadovega strupa potrdili prisotnost AntL in CRISP tudi v gadovem strupu, zato lahko predvidevamo, da je njuno sinergistično delovanje odgovorno za kardiotoksične posledice zastrupitve z gadovim strupom. S tem smo potrdili prvo hipotezo, da so tudi v gadovem strupu komponente z vplivom na srčno mišico.

Proteomska analiza gadovega strupa in primerjava s strupom modrasa je izpostavila razlike med strupoma. Pokazala je, da gadov strup vsebuje manjše število komponent oziroma da je veliko manj kompleksen. Predvsem je v njem manj nizkomolekulskih proteinov z navidezno molekulsko maso med 10 kDa in 20 kDa, kamor sodijo disintegrini in sPLA₂, in komponent z navidezno molekulsko maso nad 50 kDa, kamor sodijo hemoragične MP. Tako gadov kot tudi modrasov strup vsebujeta MP, SP, LAAO, PLTC, disintegrine, CRISP-e in sPLA₂, zato se pri pomanjkanju protistrupa proti modrasovemu strupu v praksi ob zastrupitvi pogosto uporablja protistrup, pripravljen na osnovi gadovega strupa. Primer takega protistrupa je ViperaTAB[®], ki pa ni bil učinkovit v preprečevanju širjenja edema in nevrotoksičnih znakov po zastrupitvi pacienta z modrasovim strupom [25]. Z izpostavitvijo pomembnih razlik med strupoma na molekularni ravni smo želeli prispevati k izboljšanju imunoterapije ob zastrupitvi s strupom ene ali druge kače.

Vzrok za nevrološke motnje pri zastrupitvah z modrasovim strupom so Atx, presinaptično nevrotoksične sPLA₂. Protitelesa proti Atx niso prepoznala tarč v gadovem strupu in tudi v

proteomski analizi Atx nismo zaznali, kar je v skladu s tem, da so nevrološke motnje pri zastrupitvi z gadovim strupom le redko opisane. To razloži tudi dejstvo, zakaj ViperaTAb[®] ni učinkovit pri preprečevanju nevrotoksičnih simptomov po zastrupitvi z modrasovim strupom. Atx vplivajo tudi na hemostazo, saj z vezavo na FXa inhibirajo tvorbo protrombinaznega kompleksa. S tem lahko razložimo izrazito podaljšanje protrombinskega časa v prisotnosti modrasovega strupa (za ~170 %) za razliko od gadovega, ki je ta čas skrajšal za ~20 %.

Poleg sPLA₂ na hemostazo vplivajo tudi pripadniki drugih proteinskih družin, kot so disintegrini, PLTC, LAAO, SP in MP, ki so prisotne v obeh preiskovanih strupih. Delujejo lahko na trombocite, komponente žilne stene ter koagulacijske in fibrinolizne komponente.

S preiskovanjem vpliva strupov na fibrinogen, komponento koagulacijske kaskade, smo odkrili, da je modrasov strup za razliko od gadovega razgrajeval fibrinogen intenzivneje in manj specifično. Fibrinogenolitično aktivnost imajo tako SP kot tudi MP, vendar smo dokazali, da je večina fibrinogenolitične dejavnosti v modrasovem in gadovem strupu posledica proteinov iz družine MP. Predstavnikov MP je v modrasovem strupu več, posebej patofiziološko pomembna je večja vsebnost hemoragičnih MP, ki so predvsem pripadnice P-III razreda. Le-te so vzrok za bolj izrazito hemoragijo in večje širjenje edema v okolici modrasovega ugriza. Nižja koncentracija P-III MP v gadovem strupu posledično vpliva na nižjo vsebnost njihovih protiteles v ViperaTAb[®], kar ga dela manj učinkovitega pri preprečevanju širjenja edema.

Pomembna razlika v patofiziološkem delovanju strupov obeh kač je v inhibiciji agregacije in aglutinacije trombocitov. V prisotnosti modrasovega strupa je prišlo do skoraj popolne inhibicije obeh procesov, medtem ko je bila inhibicija pri gadovem strupu šibkejša. Klasičnih PLTC, ki so disulfidno vezani $\alpha\beta$ -heterodimeri velikosti med 25 in 50 kDa, nismo zasledili v gadovem strupu. To je verjeten vzrok za veliko slabšo inhibicijo aglutinacije trombocitov z gadovim strupom kot z modrasovim. PLTC, ki jih je v modrasovem strupu veliko, so odgovorni za povzročitev trombocitopenije, zmanjšanja števila trombocitov v krvi, ki so na voljo za hemostatski odziv, hkrati pa to poveča možnost nastanka krvavitev. Opaženo je bilo, da je bilo pri zdravljenju resnejših zastrupitev z modrasovim strupom s ViperaTAb[®] potrebno večkratno dajanje protistrupa, saj se trombocitopenija, po krajšem izboljšanju, povrne [25]. Pri učinkovitosti protistupa je pomembno, kateri fragmenti protiteles se uporabijo in kakšen je način injiciranja protistrupa. Konkretno, ViperaTAb[®] vsebuje Fab fragmente, ki jih injicirajo intravensko, zato se hitro izločijo in je njihov učinek kratek. Poleg tega, je pa pomembno tudi dejstvo, da vsebuje protistrup, ki je pripravljen na osnovi gadovega strupa, premalo protiteles proti PLTC, ki bi preprečila trombocitopenijo v primeru zastrupitve z modrasovim strupom.

S tem smo izpostavili glavne vzroke za manj uspešno zdravljenje modrasovega strupenega ugriza s protistrupom, pripravljenim na osnovi gadovega strupa, in potrdili prvo hipotezo, ki trdi, da lahko proteomska primerjava strupov izboljša seroterapije. Potrdili smo vse tri hipoteze, zastavljene v doktorski disertaciji.

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Biografija

Avtor doktorske disertacije pod naslovom Komponente modrasovega strupa z vplivom na srčno-žilni sistem je Zorica Latinović, rojena 30. 11. 1989 v Ljubljani, Slovenija. Končala je Osnovno šolo Bičevje in Gimnazijo Vič v Ljubljani. Leta 2008 se je vpisala na Fakulteto za kemijo in kemijsko tehnologijo v Ljubljani (Univerza v Ljubljani), smer Biokemija. Fakulteto je zaključila leta 2014 z zagovorom diplomske naloge z naslovom Primerjalna proteomika strupov gada in modrasa ter antivenomika modrasovega strupa. V toku diplomskega študija je prejela Zoisovo štipendijo za nadarjene študente. Istega leta se je vpisala na doktorski študij na Mednarodni podiplomski šoli Jožefa Stefana, kjer je delala pod okriljem mentorja prof. dr. Igorja Križaja na Odseku za molekularne in biomedicinske znanosti na Inštitutu »Jožef Stefan«.

Glavna usmeritev njenega doktorskega študija je raziskovanje makromolekul, prisotnih v modrasovem ter gadovem strupu. Bolj podrobno se je osredotočila na proteine, ki delujejo na različne komponente srčno-žilnega sistema. Za namen podrobnega raziskovanja kardiotoksičnih in hemostatsko aktivnih molekul jih je bilo treba sprva izolirati iz KS, kar je opravila s pomočjo različnih kromatografskih tehnik (gelska kromatografija, visoko-tlačna kromatografija, ionska-izmenjevalna kromatografija itd.). Pri biokemijski karakterizaciji je posegala po metodah, kot so: de-glikozilacija, N-terminalno sekveniranje, MS in 2DE. Pri določanju primarnih tarč hemostatsko aktivnih komponent je delala poskuse v plazmi ter na *in vitro* sestavljenih kompleksih koagulacije.

Med doktorskim študijem je bila od januarja 2016 do junija 2016 na Univerzi v Singapurju. Na Odseku za biološke znanosti je pod vodstvom prof. Dr. R. Manjunatha Kini pridobivala nove izkušnje na področju karakterizacije živalskih toksinov.

Udeležila se je 12. Srečanja Slovenskega biokemijskega društva z mednarodno udeležbo, kjer je osvojila prvo mesto za poster.