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Use of MALDI-TOF mass spectrometry for specificity studies of biomedically important proteases

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Abstract. Proteases play crucial role starting from fertilization until to cell death. Our studies of the two Viperidae venoms (Levantine viper *Vipera lebetina*, Common viper *Vipera berus)* have demonstrated the existence of biomedically important proteases, both coagulants and anticoagulants that may be useful as diagnostic tools or potential therapeutics. We showed that venoms of both snakes contain: (i) metalloproteases and serine proteases that degrade fibrinogen, but not fibrin; (ii) factor X activators (VLFXA, VBFXAE); (iii) bradykinin-releasing serine proteases. Additionally *Vipera lebetina* snake venom contains thrombolytic fibrin degrading metalloenzyme (lebetase), HUVEC cell apoptosis inducing metalloprotease (VLAIP), factor V activator (VLFVA), thermostable β-fibrinogenase and α-fibrinogenase which has no homolog among known serine proteases. We examined the activity of snake venom proteases against bradykinin, substance P, insulin B-chain and 6–10 amino acid residues containing peptides synthesized according to potential cleavage regions of fibrinogen, factor X, factor IX, factor V, α_2-macroglobulin bait region and pregnancy zone protein (PZP). We used MALDI TOF mass spectrometry technique for the discovery and identification of peptides released by protease hydrolysis. The sensitive and quick MALDI-TOF mass spectrometry methodology allows us to obtain the primary information about the substrate specificity of different proteases against various peptides and proteins.

1. Introduction

Snake venoms, particularly those belonging to the Crotalidae and Viperidae families, contain a number of proteases that induce alterations in the blood coagulation cascade [1,2,4–6,8,9,23,24]. Proteases so far isolated from snake venoms are usually divided into two groups: (i) metalloproteases which need Ca^{2+} or Zn^{2+} (or both) for their hydrolytic activity and are inhibited by metal chelating agents (factor X activator, prothrombin activator, $\alpha(\beta)$-fibrinogenases, hemorrhagic proteases), (ii) serine proteases (factor V activator, protein C activator, plasminogen activator, kinin-releasing and thrombin-like enzymes, β-fibrinogenases). Recent studies revealed that some snake venoms contain factor X activators and prothrombin activators that are serine proteases [8]. Highly toxic hemorrhagic proteases degrade mammalian tissue proteins in a non-specific manner. A number of venom proteases cleave plasma proteins in relatively specific manner.

Zinc metalloproteases are widely occurring and participate in a number of important biological, physiological and pathophysiological processes (hemorrhage, fertilization, thrombolysis, cancer metastasis, etc.). Because of the possible therapeutic and diagnostic role of snake venom proteases, these enzymes merit further investigation. *Vipera lebetina* (Levantine viper) is a snake found in South-East parts of Europe, in South-West Asia and in North-West Africa. *Vipera berus berus* snake occurs in the whole

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of Europe and in Asia. The venoms of these snakes contain various proteases [11–22], which act on coagulation through both pro- and anticoagulant mechanisms and also proteases acting on extracellular membranes causing hemorrhage.

V. lebetina and *V. berus berus* venoms contain different metalloproteases [12,13,16,18,20,22]. Snake venom metalloproteases have homologous sequences and typical active site structure for reprolysins HEXXHXXGXXH [1]. The primary structure of lebetase shows extensive sequence homology with fibrolase and with several other small snake venom metalloproteases [16]. However, the substrate specificity of snake venom proteases is rather different. Lebetase, a metalloprotease with thrombolytic activity, is a direct-acting fibrinolytic agent, as it acts *via* direct cleavage of fibrin, not by plasminogen activation. Lebetase has the typical active site for reprolysins. The enzyme readily hydrolyzes the Aα chain and more slowly the Bβ chain of fibrinogen. It was demonstrated that lebetase cleaves the "bait" region in α_2-macroglobulin and hydrolyzes pregnancy zone protein (PZP) [10].

V. lebetina and *V. berus berus* venoms contain proteases that are able to activate factor X [13,20]. During physiological haemostasis, factor X can be activated by factor IXa, requiring Ca^{2+}, phospholipid, and factor VIIIa, or by factor VIIa requiring Ca^{2+} and tissue factor. Factor X is activated to the serine protease factor Xa. The activation results from the cleavage of the Arg^{52}–Ile^{53} bond in the heavy chain of human factor X and release of 52-residue activation peptide [8,25].

The alkaline serine protease from *V. lebetina* venom – a nonfibrinolytic α-fibrinogenase – has a unique specificity hydrolyzing casein but not arginine esters [14]. The enzymes degrading the β-chain of fibrinogen without fibrinolysis belong to serine proteinases. *Vipera lebetina* β-fibrinogenase is a typical representative of arginine esterases without caseinolytic activity [14]. Factor V activator from *V. lebetina* venom resembles the activator from *Vipera russelli* venom [15,17].

Due to potential use of snake venom proteases as diagnostic and thrombolytic agents, it is important to know their broader specificity against biologically active proteins and peptides. In this report we have studied the specificity of *V. lebetina* and *V. berus berus* proteases. The substrates used were oxidized insulin B chain, bradykinin, substance P and 6–10 amino acid residues containing peptides synthesized according to the literature provided protease cleavage regions in proteins such as human factor X, factor IX, factor V, fibrinogen, α_2-macroglobulin and pregnancy zone protein. The sensitive and quick MALDI-TOF mass spectrometry methodology has led us to use this technology to obtain the primary information on the enzyme cleavage sites in peptides.

2. Materials and methods

2.1. Materials

Bradykinin, Lys-bradykinin and substance P were purchased from Serva (Heidelberg, Germany). Ferulic acid, 2,5-dihydroxybenzoic acid (DHB), human factor X and fibrinogen were from Sigma (St. Louis, MO, USA), benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide hydrochloride (S-2222) from Chromogenix (Mölndal, Sweden). ZipTipC18 and C4 were from Millipore Corporation. All other reagents used were of analytical grade.

2.2. Purification of proteases

The purification of lebetase [18], VLFXA [20], bradykinin-releasing enzymes [11,19], VBFXAE [13] have been described in our previous studies. VLAIP, VBFXAEI and VBFXAEII were purified using gel

filtration, ion-exchange and affinity chromatography (unpublished). Enzymes were analyzed by SDS-polyacrylamide gel electrophoresis, isoelectric focusing, HPLC and MALDI TOF mass spectrometry.

2.3. Fibrinogen degradation

Specific cleavage of fibrinogen was shown on 5–15% polyacrylamide gels. One half ml of 1% fibrinogen solution was incubated with 25 μg of enzyme at 37°C in 0.05 M Tris-saline buffer (pH 7.4). At various time intervals, 50 μl aliquots were withdrawn and added to 50 μl of denaturing solution (10 M urea, 4% SDS, 4% 2-mercaptoethanol). The samples were reduced and denatured overnight at 37°C before being electrophoresed.

2.4. Enzyme activities

Caseinolytic activity was assayed by the method of Kunitz as modified by Mebs [7]. Bradykinin-releasing activity was determined using heated human plasma (56°C, 3 h) as substrate. Plasma was centrifuged and dialysed against 0.05 M ammonium acetate. 5–10 μl of enzyme solution (1 mg/ml) was added to 200 μl of treated plasma containing 1 mM o-phenanthroline and the mixture was incubated at 37°C for 20 min. The mixture was ultrafiltrated (Ultra spin ultrafilter, cut-off 10 000). The kinin was detected in filtrate by MALDI-TOF mass spectrometry.

The activation effect of factor X by VLFXA was measured by the amidolytic activity of the factor Xa that was formed according to the method described by Hofmann and Bon [3]. Bovine or human factor X was used as substrate for VLFXA. Factor Xa activity was determined with S-2222 by recording the liberation of p-nitroaniline at 405 nm. The degradation products of human factor X treated with VLFXA were detected by MALDI TOF MS.

2.5. SDS-PAGE

SDS-polyacrylamide gel electrophoresis was carried out in 10% and 12.5% gels and gradient gels. The following molecular mass indicators were used: bovine serum albumin (67 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), cytochrome C (12.3 kDa). Staining was performed with Coomassie Brilliant Blue R250.

2.6. Peptide synthesis

All peptides were synthesized at the 100 μmol scale on Applied Biosystems 431A Peptide Synthesizer using BOC (t-butyl-oxycarbonyl) chemistry as suggested by the manufacturer.

The purity of peptides was assessed by analytical reverse phase – high performance liquid chromatography (HPLC) and MALDI-TOF mass spectrometry.

2.7. MALDI-TOF mass spectrometry

The MALDI mass spectra were measured with a home-built gridless time-of-flight MALDI mass spectrometer designed for maximum flexibility in use (National Institute of Chemical Physics and Biophysics). Before MALDI-TOF analyses in some cases protein samples were purified from salts using ZipTip C_4 or ZipTip C_{18} according to Millipore instructions. The matrix used for MALDI-TOF MS analyses of proteases, factor X and cleavage products of factor X was ferulic acid. Cytochrome C and bovine carbonic anhydrase was used for mass calibration.

2.8. Monitoring of the enzymatic reaction. MALDI-TOF mass spectrometry of peptides

Peptide and peptide cleavage analyses by MALDI-TOF MS have been previously reported [26]. All peptide solutions were directly prepared in 0.1 M NH_4HCO_3, at concentrations of about 1–5 mg/ml, and kept frozen at -20°C until use. The enzymatic hydrolysis of peptides was carried out in 0.1 M NH_4HCO_3, at 37°C in an eppendorf tube. In a typical experiment, 100 μl of 0.1 M NH_4HCO_3 solution of substrate (1 mg/ml) in an eppendorf tube was thermally equilibrated to 37°C in the thermostated rack. The reaction was started by addition of 15 μl of enzyme solution (1 mg/ml in 0.1 M NH_4HCO_3). At predetermined time intervals (5 min, 0.5 h, 20 h), the aliquot (10 μl) was diluted with 100 μl of H_2O and 2 μl of 6 N HCl was added to stop the reaction. One half μl of diluted mixture was used for MALDI-TOF mass spectrometry analysis. The matrix used for peptide analyses was 2,5-dihydroxybenzoic acid (DHB). 10 mg of DHB was dissolved in 1 ml of a 1 : 1 mixture of 0.1% trifluoroacetic acid and acetonitrile for sample preparation. One half μl of this mixture was deposited on a stainless steel probe tip, mixed there with 0.5 μl of reaction mixture of peptides and enzyme and allowed to dry at room temperature. External mass calibration was accomplished by using as peptide standards, substance P and Lys-bradykinin.

For peptide analysis the mass spectrometer was operated in reflectron mode with 2.6 kV pulsed extraction, 14.6 kV total acceleration voltage and 500 ns delay between laser and extraction pulse. A double multichannel plate detector was used for ion detection.

3. Results and discussion

Viperidae venoms contain a lot of serine and metalloproteases. From *V. lebetina* venom following proteases were purified: fibrinolytic enzyme lebetase [18], factor X activator (VLFXA) [20], factor V activator (VLFVA) [17], bradykinin releasing enzymes [19] and HUVEC (human umbilical vein endothelial cell) apoptosis inducing protease (VLAIP); from *V. berus berus* venom: factor X activating proteases (VBFXAE [13], VBFXAEI, VBFXAEII) and bradykinin releasing enzymes [11]. Molecular masses of proteases, detected by MALDI TOF MS and SDS–PAGE, are provided in Table 1. Mass spectrum of VLAIP is given in Fig. 1. All proteases (except lebetase) are glycosylated [11–14,17].

Specificity of *V. lebetina* and *V. berus berus* venom metalloproteases was studied against different proteins and peptides. The most popular substrate for characterization of proteases is oxidized insulin B chain. In our previous studies [12,13,18] we used amino acid analysis of proteolysis cleavage products of insulin B chain. The cleavage of insulin B chain by VLAIP, VBFXAEI and VBFXAE II was investigated using MALDI-TOF MS that is more effective and quick method for detection of peptide fragments. Lebetase [18], VBFXAE [13], VBFXAEI, VBFXAEII cleave Ala^{14}–Leu^{15} and Tyr^{16}–Leu^{17} bonds (Tables 2, 3; Fig. 2). VLAIP cleaves only Ala^{14}– Leu^{15} bond (Fig. 3) and VLFXA does not hydrolyze insulin B-chain at all.

Substance P was cleaved by lebetase mainly at Pro^{4}–Gln^{5} position [26]. The cleavage of substance P by VLAIP is rather different (Table 2). Lebetase cleavage sites in the "bait" region of α2-macroglobulin and PZP were determined by Edman degradation analysis of the digestion products [10]. Corresponding peptide fragments were used as substrates for lebetase [26] and VLAIP. Both enzymes cleave the same bonds in these peptides (Table 2).

Many snake venom proteases have fibrinogenolytic activities. Fibrinogen has often been used as substrate for various venom proteases [1,5,18]. *V. lebetina* and *V. berus berus* venom contain proteases (lebetase, VLAIP, VBFXAE, VBFXAEI, VBFXAEII) that cleave mainly Aα-chain of fibrinogen and more

Table 1
Molecular masses of *V. lebetina* and *V. berus berus* proteases

Enzyme	Nonreduced Da	Reduced Da
Lebetase	22912 [22]*	23700 [18]
VLAIP	106000*	60000
VLFXA	86500–91000 [20]*	57500; 17400; 14500 [20]
VLFVA	28400 [17]*	30000 [17]
β-fibrinogenase	42200 [14]*	52500
α-fibrinogenase	31100 [14]*	37000
VBFXAEI	97500	56900; 16800; 12000
VBFXAEII	125900	59300
VBFXAE	38000 [13]	38000 [13]

*Detected by MALDI-TOF MS.

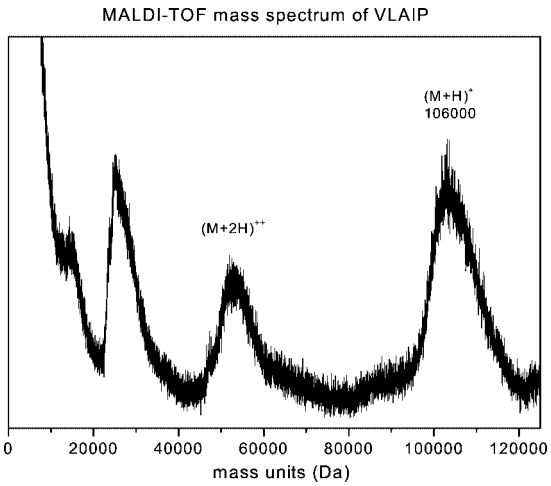

Fig. 1. MALDI-TOF mass spectrum of VLAIP. $(M + H)^{+}$ was indicated as the molecular ion while $(M + 2H)^{++}$ as double charged ions. Matrix was ferulic acid.

slowly Bβ-chain. *V. lebetina* venom α-fibrinogenase degrades only Aα-chain [14]. None of them attacks γ-chain of fibrinogen. *V. lebetina* venom contains thermostable β-fibrinogenase that preferably cleaves β-chain of fibrinogen [14]. We have shown that cleavage patterns of fibrinogen treated with venom proteases are different [14,18]. We synthesized 6–10 amino acid containing peptide fragments according to fibrinogen Aα-chain cleavage site regions for various venom metalloproteases (Lys^{413}–Leu^{414}, Pro^{516}–Met^{517}) [26]. These peptides were used as potential substrates for fibrinogenolytic metalloproteases. Lebetase cleaved the peptide EYHTEKLVTS at position Lys^{6}–Leu^{7} and the peptide FFSPMLGE at position Pro^{4}–Met^{5} [26], the same positions are attacked also by VLAIP but the reaction is considerably slower (Table 2). VBFXAEI cleaved Lys^{6}–Leu^{7} bond and more slowly Tyr^{2}–His^{3} bond in EYHTEKLVTS, VBFXAEII catalyzed only the cleavage of Lys^{6}–Leu^{7} bond (Table 3). Neither *V. berus berus* enzyme cleaved the peptide FFSPMLGE.

The purified factor X activator (VLFXA) from *V. lebetina* venom had no effect on fibrinogen, prothrombin, plasminogen, indicating that the activation of factor X was specific [20]. The effects of VLFXA, VBFXAE, VBFXAEI and VBFXAEII on human factor X were studied by measuring the ami-

Table 2
Hydrolysis of peptide substrates by lebetase and VLAIP

Name of substrate	Lebetase cleavage sites [26]	VLAIP cleavage sites
Oxidized insulin B chain		
FVNQHLC(SO_3H)GSHLVEA^{14}L^{15}-	Ala^{14}–Leu^{15}	Ala^{14}–Leu^{15}
Y^{16}L^{17}VC(SO_3H)GERGFFYTPKA	Tyr^{16}–Leu^{17}	
Substance P		
RPKPQQFFGLM	***Pro^4–Gln^5**	Gln^5–Gln^6
	Gly^9–Leu^{10}	Phe^7–Phe^8
	Pro^2–Lys^3	Gly^9–Leu^{10}
Bradykinin		
RPPGFSP↓FR	Pro^7–Phe^8	Pro^7–Phe^8
Fibrinogen fragment 406–417		
HTEK↓LVTS	Lys^4–Leu^5	Lys^4–Leu^5
Fibrinogen fragment 513–520		
FFSP↓MLGE	Pro^4–Met^5	Pro^4–Met^5
α_2-M fragment 693–700		
GHAR↓LVHV	Arg^4–Leu^5	Arg^4–Leu^5
α_2-M fragment 676–684		
GPEG↓LRVGF	Gly^4–Leu^5	Gly^4–Leu^5
PZP fragment 686–693		
PYVP↓QLGT	Pro^5–Gln^6	Pro^5–Gln^6

*Pro↓**Gln** is the main cleavage site (after 5 min hydrolysis) of substance P by lebetase.

Table 3
Cleavage sites of peptides by factor X activating enzymes from *Vipera berus berus* venom

Substrate	VBFXAEI	VBFXAEII	VBFXAE
Oxidized insulin B-chain			
FVNQHLC(SO_3H)GSHLVEA^{14}L^{15}-	**Ala^{14}–Leu^{15}**	**Tyr^{16}–Leu^{17}**	**Tyr^{16}–Leu^{17}**
Y^{16}L^{17}VC(SO_3H)GERGFFYTPKA	**Tyr^{16}–Leu^{17}**	Ala^{14}–Leu^{15}	Ala^{14}–Leu^{15}
Factor X fragment	**Arg^5–Ile^6**	**Leu^3–Thr^4**	**Leu^3–Thr^4**
NNL^{3}T^{4}R^{5}I^{6}VGG	Leu^3–Thr^4	Arg^5–Ile^6	Arg^5–Ile^6
Factor IX fragment	No cleavage	Arg^5–Val^6	Not detected
DFTR^{5}V^{6}VGG			
Fibrinogen Aα-chain fragment	**Lys^6–Leu^7**	**Lys^6–Leu^7**	Not detected
EY^{2}H^{3}TEK^{6}L^{7}VTS	Tyr^2–His^3		

Preferably cleaved bonds are **bold**.

dolytic and the coagulant activities of the activated factor X (factor X_a). The activators convert the inactive factor X in the presence of Ca^{2+} ions to the active form Xa which activity was detected using substrate S-2222 in a complex two-stage reaction. The factor X activating enzymes themselves have no amidolytic activity against factor Xa substrate S-2222. To simplify the localization of factor X activators in the process of purification, 6–9 amino acid residues containing peptide fragments (TRIVGG, LTRIVGG and NNLTRIVGG) corresponding to the physiological cleavage region of human factor X were synthesized. VLFXA cleaved Arg–Ile bond in these peptides whereas the peptide NNLTRIVGG

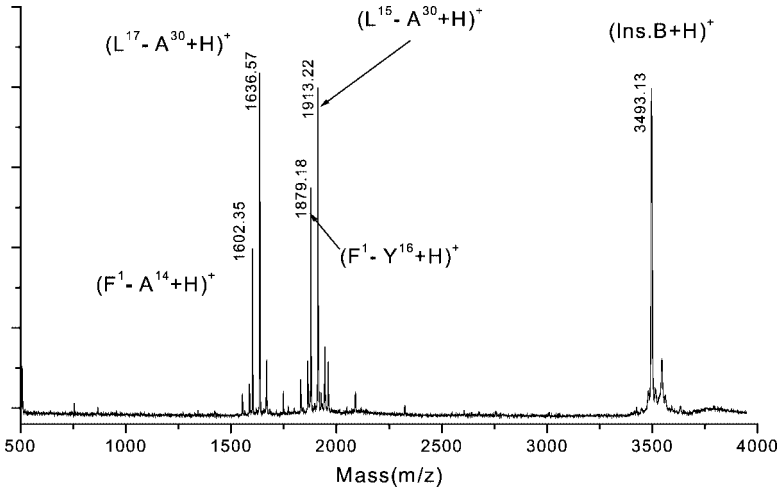

Fig. 2. MALDI-TOF mass spectrum of cleavage products of oxidized insulin B chain after 2 hours treatment with VBFXAE. Matrix was DHB.

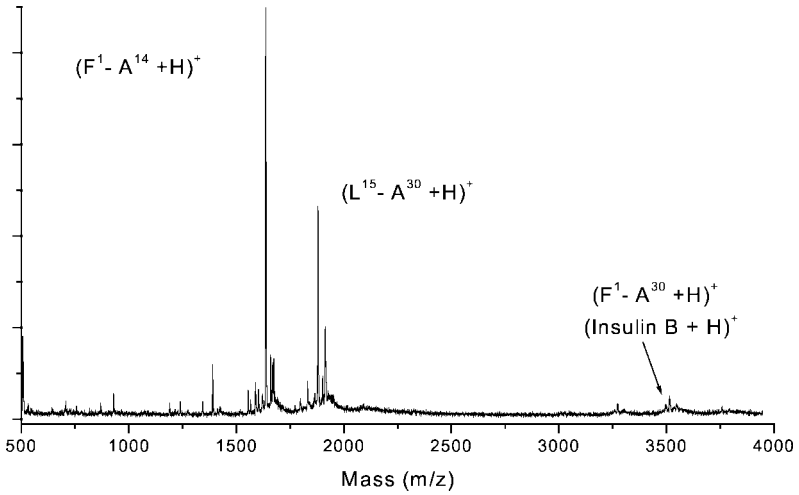

Fig. 3. MALDI-TOF mass spectrum of cleavage products of oxidized insulin B chain after 20 hours treatment with VLAIP. Matrix was DHB.

was the best substrate (Fig. 4). VLFXA may also activate factor IX while it hydrolyzed 9 amino acid residues containing peptide fragment NDFTRVVGG, synthesized according to the physiological cleavage region of human factor IX, in position Arg–Val [20].

Unlike VLFXA, *V. berus berus* venom factor X activating enzymes hydrolyze the peptide NNLTRIVGG at two positions: Arg^5–Ile^6 and Leu^3–Thr^4 whereas VBFXAEI catalyzes preferably Arg^5–Ile^6 bond and VBFXAE and VBFXAEII preferably Leu^3–Thr^4 bond (Table 3). The specificity studies of factor X activating enzymes from *V. berus berus* venom have shown that, besides factor X, these enzymes cleave other proteins such as fibrinogen, asocasein and insulin B chain. However, all three enzymes release factor X_a from human and bovine factor X, although the specific activities of *V. berus berus* venom enzymes are lower than these of RVV-X (*V. russelli* factor X activator [25]) and VLFXA. Consequently, *V. berus berus* venom factor X activating enzymes are nonspecific.

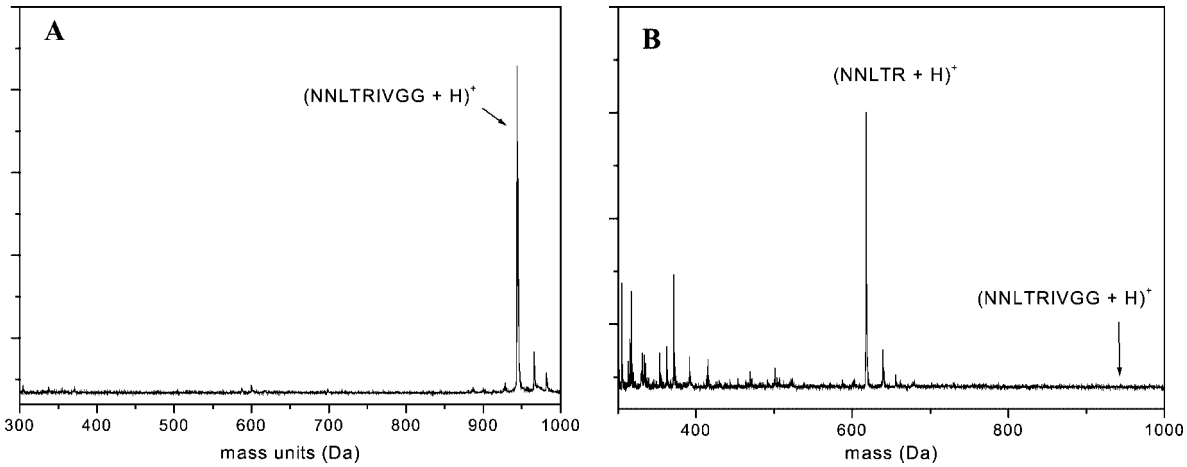

Fig. 4. (A) MALDI TOF mass spectrum of human factor X peptide fragment 48–56 NNLTRIVGG, (B) mass spectrum of cleavage products of human factor X peptide fragment after treating with VLFXA. Matrix was DHB.

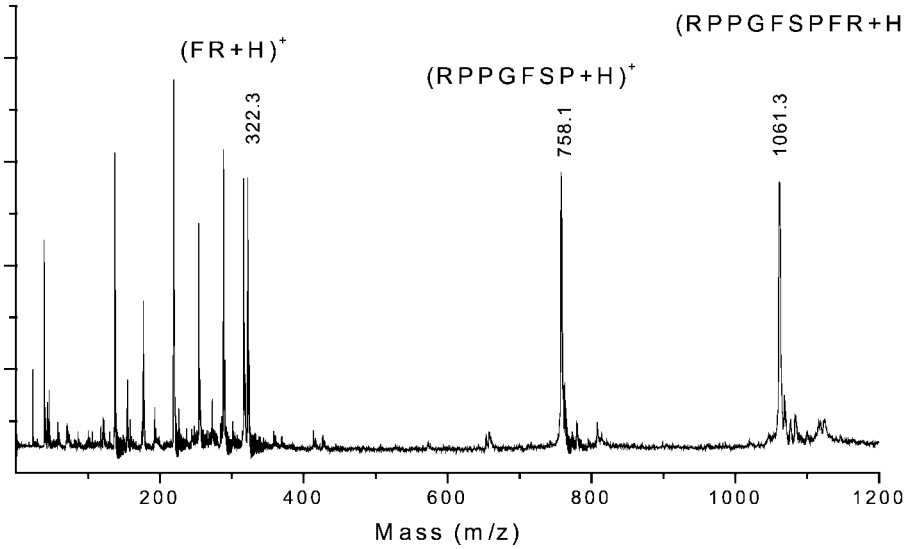

Fig. 5. MALDI-TOF mass spectrum of cleavage products of bradykinin after 5 minutes treatment with lebetase. Matrix was DHB.

VLFVA hydrolyzed human factor V peptide fragments containing 7–9 amino acids (YLRSNNG, WYLRSNNG and AWYLRSNNG), corresponding to *V. russelli* factor V activator cleavage region in human factor V. VLFVA cleaves Arg–Ser bond in these peptides [21].

We have shown that *V. lebetina* and *V. berus berus* venoms contain bradykinin-releasing serine enzymes [11,19]. MALDI TOF MS was very effective for detection of bradykinin that was liberated from human plasma after treating with bradykinin-releasing enzymes from *V. lebetina* [21] and *V. berus berus* venoms. This method successfully replaces the rat uterus test formely used for kinin detection. Bradykinin is a naturally occurring peptide that plays a role in maintenance of blood pressure. Thrombolytic enzyme lebetase cleaves bradykinin at the position Pro^{7}–Phe^{8} [26], as well as the other metal-

loprotease VLAIP does (Table 2). Cleavage of bradykinin by lebetase (an important side effect of the enzyme) destroys its biological activity. Viperidae venoms contain several serine proteases that release bradykinin and metalloproteases that cleave bradykinin, therefore the detection of bradykinin-releasing enzymes without metalloprotease inhibitors in crude venom is inaccurate.

The use of MALDI-TOF MS has several advantages over traditional methods for the elucidation of cleavage sites by proteases in peptides and proteins. MALDI-TOF MS has high sensitivity that allows the analysis of small aliquots removed from reaction mixture. Our results show that MALDI-TOF MS is a very informative tool and enables the characterization of the composition of cleaved peptides and proteins. However, as it stands now, MALDI is not quantitative enough for detecting kinetic parameters of enzyme reactions.

Acknowledgements

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