Effects of snake venom proteases on human fibrinogen chains

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Background. Proteomic approach is an effective method to study changes in human plasma proteome. Coagulopathies are commonly encountered in victims of viper envenomation which were treated with an administration of immunoglobulin. Unfortunately, this treatment shows significant risk to the patient due to an anaphylactic reaction. Since *Echis carinatus* Venom (EV) toxins mainly acts both directly and indirectly on fibrinogen, we planned to establish a suitable analysis of its beta (FIBB) e gamma (FIBG) chains. This study will help us to understand the mechanism of envenomation and to find alternative treatments other than the common treatment with the administration of IgG.

Study design and methods. We evaluated the EV proteolytic activity on whole human plasma proteome from the blood of an healthy volunteer. Two-dimensional electrophoresis (2-DE) using mini-gel was performed to analyse EV effects on the differents fibrinogen chains.

Results. Changes in whole plasma proteome were focused on fibrinogen beta and gamma chains after EV incubation. Protein spots were detected and analyzed using ImageMaster 2D Platinum software. Results were represented as mean±standard deviation (mean±SD) with $p<0.05$ as a statistically significant value. 2-DE gel analysis showed that some spots of FIBB disappeared and some spots of FIBG decreased.

Conclusion. We found that the proteomic approach is a valid method in studying in-depth causes of different diseases, in particular those are involved in coagulopathies linked with proteins like fibrinogen from victims of viper envenomation.

Key word: proteome, plasma, envenomation, proteolysis

Introduction

Venoms from *Viperidae* family contain a large variety of proteins and peptides affecting the haemostatic system. Venom components can act as procoagulants, causing the activation of the coagulation system[1]. This does not result in massive thrombosis and consequent embolic diseases because snake venom presents thrombolytic activity. It was demonstrated in canine model and it revealed that the activity of enzymes functioned independently of the native fibrinolytic system and produced rapid and consistent thrombolysis[2]. Thus it is important to consider the proteolytic events of the proteinases on plasma proteins. Based on sequence, snake venom proteinases have been classified into various families, mainly serine proteases and metalloproteinases[3]. Direct fibrin(ogen)olytic metalloproteases degrade preferentially the Aα-chain of fibrinogen followed by the β-chain. The enzymes degrading the β-chain without fibrinolysis belong to the serine proteases group[4-5]. Some metalloproteases have fibrinogenolytic or fibrinolytic activities and are named fibrinogenases. These enzymes have been classified as α, β and γ-fibrinogenases based on their specificity for cleaving

fibrinogen polypeptide chains[6-9]. Fibrinogen molecules are structures consisting of two outer D domains, each connected by a coiled-coil segment to a central E domain. They are comprised of two sets of three polipeptide chains termed Aα, Bβ and γ.

These chains are joined together within its N-terminal E domain by five symmetrical disulfide bridges[10]. Echis carinatus Venom (EV) contains two metalloproteinases which are prothrombin activator: ecarin (EC) and carinactivase (CA)[11].

EC action on prothrombin is independent of calcium, phospholipids and factor V. It is the primary reagent in the Ecarin clotting time test[12]. CA is strongly dependent on calcium ions for the activation of prothrombin[13-14]. Using venom from *Echis carinatus* we regularly test its activity on whole human plasma focusing the EV effects on the coagulation process mainly represented by the breakdown of the fibrinogen[15].

Materials and methods

The study was conducted in the Department of Internal Medicine, Endocrine-Metabolic Sciences and Biochemistry at the University of Siena over a period of 20 months from February 2008 to October 2009. We prepared the human plasma sample from the blood of an healthy volunteer. We incubated human plasma with *Echis carinatus* venom (EV) that was from Sigma. 2-DE analysis and proteins identification were performed using the EttanDalt Six Electrophoresis system, Ettan IPGphor, Multi Temp III, Immobiline_DryStrip 7 cm pH 3-10 NL, Immobilized pH gradient (IPG) buffer pH 3-10 NL, DryStrip Cover Fluid and Agarose M that were from Amersham Biosciences. Other materials used to prepare the electrophoresis experiments such as ammonium persulphate (APS), 1,4-Dithio-DL-threitol (DTT), iodoacetamide (IAA), sodium thiosulphate, α-cyano-4-hydroxycinnamic acid (CHCA), acetonitrile (ACN), trifluoracetic acid (TFA) and laemmli sample buffer were from Sigma.

Each sample was carried out in triplicate under the same conditions. We detected proteins in gels by silver staining. After scanning the gels, using Image Scanner, protein spots were detected and analysed using ImageMaster® 2D Platinum software.

After tryptic digestion, each spot digested was analysed using ETTAN MALDI-TOF and mass fingerprinting searching was carried out in Swiss-Prot/TREMBL and NCBInr databases using MASCOT, an on-line available software.

Preparation of venom

Crude EV (common name: saw-scaled viper, family: Viperidae, subfamily: Viperinae, genus: Echis, species: carinatus) subspecies: sochureki (taxonomy ID:124223) was suspended in physiological solution at the calculated concentration for the treatment.

Preparation of human plasma

Blood from a healthy human volunteer was collected in two test tubes containing citrate as anticoagulant. The erythrocytes were removed and plasma from one test tube was then pooled and stored at –80 °C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

In vitro activity of EV was assayed by incubating 25 µL of EV (0.02 µg/µL) with 25 µL plasma (0.6 µg/µL) at a ratio of 1:30, with 50 µL of incubation buffer containing 50 mM Tris-HCl, 140 mM NaCl, 5 mM $CaCl_2$ pH 7.4 for 0, 30, 60 min at 37 °C.

The optimal EV concentration was performed by mixing 75 µL of incubation buffer with 25 µL of EV (0.02 µg/µL) for 60 min at 37 °C. The mixtures and a low molecular weight standard (LMW) were then separated on SDS-PAGE (12%) which standardize the optimal ratio between plasma and EV for 2-DE, without its bands being evidenced by silver stain. The bands were automatically detected and analyzed using TotalLab software. Band intensity was expressed as a proportion of the total protein intensity detected for the entire lane.

This allowed normalization of band intensity which was used to compare band measurements in different lanes.

2-DE analysis

Each plasma sample containing 60 µg of protein as determined by the Bradford assay[27] was prepared as follows: control plasma samples were incubated for 60 min at 37 °C with an incubation buffer containing 50 mM Tris-HCl, 140 mM NaCl, 5 mM $CaCl_2$ pH 7.4; treated plasma sample was incubated for 60 min at 37°C with 1 µg of EV at a ratio of 1:30

and with incubation buffer. Samples were denatured with 10 µL of a solution containing 10% SDS, 2.3% DTT, heated to 95 °C for 5 min and then cooled.

The sample was then combined with 350 µL of solubilising buffer containing 8 M urea, 2% CHAPS, 0.3% DTT, 2% IPG buffer and a trace of bromophenol blue and loaded into 7 cm IPG strips 3-10 NL on an Ettan IPGphor apparatus system and rehydrated for 7 h. IEF was carried out for a total of 32 kV·h. After focusing, the strips were first equilibrated with equilibration buffer containing 50 mM Tris-HCl pH 8.8, 6 M urea, 2% w/v SDS, 30% v/v glycerol, and 1% w/v DTT for 15 min, then were equilibrated again with the same equilibration buffer except containing 4% w/v IAA instead of DTT and a trace of bromophenol blue.

The strips were then washed for a further 10 min with Tris-glycine buffer. The second dimension was performed on an EttanDalt Six Electrophoresis system. IPG strips and a wide range molecular weight standard (WMW) were embedded at the top of a 1.5 mm thick vertical polyacrylamide gradient gel (8-16%T) using 0.5% w/v agarose and run at a constant current of 40 mA/gel at 20°C. Each sample was carried out in triplicate under the same conditions. The exposure time for silver staining was also optimized to avoid overexposure of some gels with respect to others.

Image and statistical analysis

Protein spots were automatically detected and analysed using ImageMaster 2D Platinum software. Spot volume was expressed as a ratio of the total protein volume (%Vol) detected from the entire gel. This allowed normalization between gels. One control gel and one treated gel were used as the reference gels for comparison. The background was subtracted from all gels using the average-on-boundary method. Relative mass (M_r) in kilodalton (KDa) and isoelectric point (p*I*) were also calculated for each spot. Only spots appearing in all three gels were matched with those of the reference gel. We considered quantitative differences only those with of at least two-fold variation in spot relative volume %V. Statistical analysis of protein variation was performed with the *t*-test using the GraphPad Prism software. Results were expressed as means±SD and $p<0.05$ was considered statistically significant.

Tryptic digestion and protein identification MALDI-TOF MS

After revelation by the MS compatible staining protocol in 2-DE, electrophoretic spots were excised, destained and dehydrated with ACN for subsequent rehydratation with trypsin solution. Tryptic digestion was carried out overnight at 37 °C. Each protein spot digest (0.75 µL) was spotted onto the MALDI instrument target and allowed to dry. Then 0.75 µL of the instrument matrix solution (saturated solution of CHCA in 50% ACN and 0.5% v/v TFA) was applied to dried sample and dried again. Mass spectra were obtained using an ETTAN MALDI-TOF mass spectrometer from Amersham Biosciences (Uppsala, Sweden). After tryptic peptide mass acquisition, mass fingerprint searching was carried out in Swiss-Prot/TREMBL and NCBInr databases using MASCOT (Matrix Science, London, UK, http://www.matrixscience.com) an on-line-available software. A mass tolerance of 100 ppm was allowed and only one missed cleavage site was accepted. Alkylation of cysteine by carbamidomethylation was assumed as fixed modification, while oxidation of methionine was considered a possible modification. The criteria used to accept identifications included the extent of sequence coverage, number of matched peptides and probabilistic score.

Results

Figure 1 showing the experimental procedure was designed mainly to define the condition to perform SDS-PAGE experiment with plasma and venom at the optimal quantities, ratio and incubation time. This study shows that the optimal quantity of plasma for SDS-PAGE is 0,6 µg showing clearly changes in bands. The optimal quantity of EV is 0,02 µg; the amount of venom which does not influence plasma pattern after silver staining. SDS-PAGE of plasma incubated with EV at 0, 30, 60 min shows changes in bands indicated by arrows and progressive numbers. From our results, we considered 60 min as the optimal incubation time because it revealed a considerable number of changes. The gel was analyzed by TotalLab software and significant protein changes are shown by histograms in percentage volume (%V) values.

Figure 2 shows 2-DE of control plasma, treated plasma and venom alone. 2-DE was performed to better evidence changes in fibrinogen chains due to

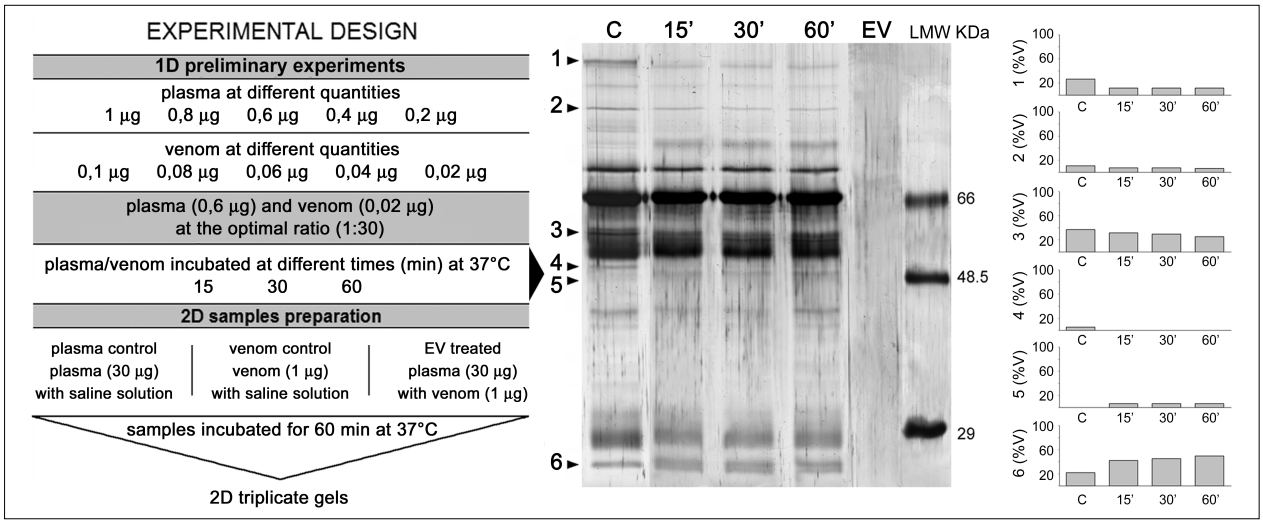

Figure 1 - SDS-PAGE preliminary procedure to standardize the 2-DE experiment.

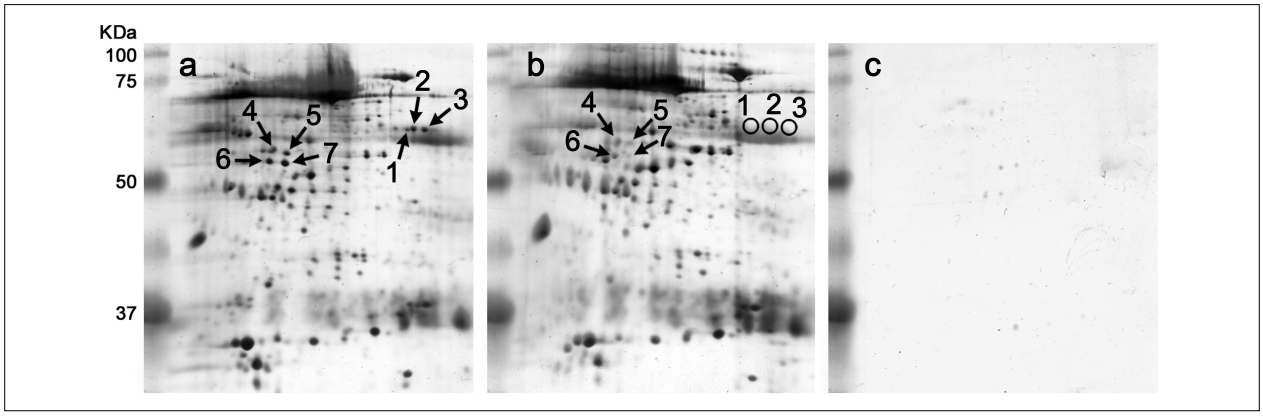

Figure 2 - 2-DE maps of control plasma, EV treated plasma and EV alone.

the EV proteolytic activity. Clearly, the pattern of the plasma treated with EV shows many evident changes with respect to the control plasma refer to other plasma proteins not here investigated. Figure 2 shows the representative proteomic maps of control and treated plasma with all the identified differences highlighted in fibrinogen protein. In the control map represented in image (a) and the treated one, in image (b), we have reported the identified quantitative and qualitative variations of FIBB and FIBG: spots indicated by black arrows and progressive numbers 1, 2, 3 represent the FIBB which disappeared after EV treatment and reported respectively by black circles in image (b) in Figure 2; while spots indicated by black arrows and progressive numbers 4, 5, 6, 7 in image (a) and in image (b) in Figure 2, represent FIBG which decreased after EV treatment. With 2-DE analysis we studied these two types of EV activity on the two chains of fibrinogen where it is evident that EV acts completely on FIBB and partially on FIBG. Image (c), in Figure 2, shows that the proteome pattern of EV does not influence the plasma proteins pattern in 2-DE as shown in SDS-PAGE experiment. An optimal EV quantity was used without its bands or spots being evidenced by silver stain.

Results were represented as mean ± SD with $p<0.05$ as a statistically significant value. 2-DE gel analysis showed that 3 spots of FIBB disappeared and 4 spots of FIBG decreased. From the 4 spots of gamma chain only 2 changed significantly.

Table I shows fibrinogen chains identified by mass spectrometry. It reports all the important information

Table I - Summary of identified proteins showing significant qualitative and quantitative variations

Spot ID	AC[a)]	Protein name	Entry name	p*I*/M_r (KDa) predicted	p*I*/M_r (KDa) experimental	Peptides matches	Sequence coverage, %	MOWSE score	M±SD (%V)	
									Control	EV treated
Qualitative variations										
1	P02675	fibrinogen beta chain	FIBB	8,54/56577	8,36/56139	19	38	188	0,53±0,12	nd
2	P02675	fibrinogen beta chain	FIBB	8,54/56577	8,54/56500	18	34	147	0,64±0,13	nd
3	P02675	fibrinogen beta chain	FIBB	8,54/56577	8,86/56410	11	27	105	0,40±0,07	nd
Quantitative variations										
4	P02679	fibrinogen gamma chain	FIBG	5,37/52106	5,68/52995	10	30	124	0,37±0,09	0,26±0,03
5	P02679	fibrinogen gamma chain	FIBG	5,37/52106	5,76/52741	13	41	173	0,31±0,04	0,19±0,09
6	P02679	fibrinogen gamma chain	FIBG	5,37/52106	5,67/51407	11	34	138	0,43±0,05	0,16±0,01*
7	P02679	fibrinogen gamma chain	FIBG	5,37/52106	5,74/51079	15	43	178	0,49±0,06	0,06±0,0007**

*a) accession numbers of Swiss-Prot. For proteins significantly decreased: * p<0.05, ** p<0.01; spot not detected are represented by nd; spot ID refers to that reported in the Figure II.*

about mass spectrometry identification such as the peptide matches, the sequence coverage and the probabilistic score obtained by using Mascot software. Each protein species is specified by a spot ID that corresponds to the number present on Figure 2. Accession number of Swiss-Prot database and protein name are also included. Predicted and experimental p*I* and M_r are reported respectively from mass spectrometry and from gels, in order to confirm the MASCOT search or to have a view of the possible post-translational modifications present.

Discussion

Since coagulation disorders involving fibrin clot is the mainly effect of EV toxicity, we carried out experiments based on EV effects on different fibrinogen chains (FIBB and FIBG) using the proteomic approach. During the coagulation the N-terminal region of each Aα chain contains the fibrinopeptide A (FPA) sequence. Thrombin cleavages FPA and initiates fibrin assembly by exposing a polymerization site contained in central E domain. Assembly of fibrin begins with non-covalent interactions between E domain and D domain sites to form end-to-middle staggered overlapping double-stranded fibrils. Fibrils also branch and undergo lateral associations to form wider fibrils and fibers. After cleavage of the fibrinopeptide B (FPB), contained in the N-terminal region of each Bβ, domains in α chains become available for self-association with other α chain domains, thereby promoting lateral fibril associations and fiber assembly. Then, factor XIII contacts γ chain domain and introduces bonds between C-terminal γ sites to rapidly form γ dimers. γ Trimers and γ tetramers form more slowly by interfibril γ chain crosslinking, and increase the resistance of the clot to fibrinolysis[16-17]. Fibrinogen is a plasma protein that interacts with integrin to mediate a variety of platelet responses including adhesion, aggregation, and clot retraction. Also the human FIBG C-terminal segment functions as the platelet integrin binding site[18-19]. The proteolysis of the fibrinogen segments can lead to the loss of its property to interact with blood cells involved in the immune/inflammatory response[20]. In our study, after mass spectrometry, we identified FIBB and FIBG which were changed after EV treatment with qualitative and quantitative variations. These variations can be linked to the balance of the equilibria between the clot formation and its dissolution, which is physiologically determined by plasmin. Its dysfunction, caused by protease activity, leads to the loss of its capacity to yield monomers that polymerize into fibrin and leads to the loss of action as a cofactor in platelet aggregation. Fibrinogen is the specific substrate of the thrombin, a serine protease and the key enzyme of blood coagulation[21]. Our work shows that it is also a substrate of proteases contained in EV which act in two modality on fibrinogen chains, reported in Table I. Other vipers, like *Trimeresurus gramineus*, contain venoms with proteases active on fibrinogen. Trimeresurus gramineus venom contains a metalloprotease known as graminelysis I. This

metalloproteinase acts, as EV, on FIBB and has a little effect on FIBG[22]. We can represent the protein-protein-protease interaction and their association with diseases as a network. These results give us new information about human plasma proteome, proteases and snake venom poison. This information can help us to understand the role of these three moieties in medical application.

Conclusion

The proteomic approach is a sensitive and valid method to study in-depth changes in blood proteins and in their shares like the chains of fibrinogen. This study can lead to the identification of therapies against the poisoning by snake venoms based on the inhibition of snake venom proteases, using more specific and selective inhibitors. These inhibitors should block the fibri(ogen)olysis. This approach can solve the problem due to the administration of IgG, avoiding all its related risks.

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