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Enzyme electrophoresis method in analysis of active components of haemostasis system

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ABSTRACT

The novel modifications of substrate-containing sodium dodecyl sulfate-polyacrylamide gel electrophoresis that can be used for the detection of proteases and its activators are reported. The protease/activator samples were separated on a protein substrate-SDS-polyacrylamide gel. To detect plasminogen activators fibrinogen and Glu-plasminogen were incorporated into the SDS-PAG followed by 1 h incubation at 37˚C in thrombin solution (1 NIH/ml). After electrophoresis the gel was stained according to the standard protocol. To detect fibrin-unspecific plasminogen activators from snake venom incubation in thrombin solution was substituted for 12 h incubation in 50 mM Tris-HCl (pH 7.4). To detect fibrinogen-degrading enzymes fibrinogen-containing gel was used. Activity of protease/activator was visualized in the gel as clear bands against the dark background. These new techniques offer several advantages including determination of the quantity and activity of t-PA and urokinase, however cannot be recommended for precise quantification of activators; the total procedure is quite quick and simple; method is convenient tool for detection of novel protein-protein interactions in haemostasis system; the sensitivity of the method is ≤0.01 IU per track.

Keywords: Substrate-Containing Electrophoresis; Enzyme Electrophoresis; Haemostasis; Proteins Activity; Proteins Identification

1. INTRODUCTION

Protein electrophoresis is a convenient approach to characterize sample composition, protein interactions, purity, molecular weight, isoelectric point, and to purify small amounts of protein for further analysis. Different modifications of this widely used method have been developed to suit a variety of purposes [1].

Erickson [2] and Brunner [3] offered fibrin autography to detect protease activators/inhibitors previously fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-***PAGE***). After separation the proteins and the substrate were transferred electrophoretically into the fibrin indicator gel. The positions of activators/inhibitors were revealed by the formation of contrast fibrinolytic or lysis-resistant zones.

Hanspal *et al*. [4] described a technique to detect the activity of protease inhibitors present in enzyme substrate-containing sodium dodecyl sulfate polyacrylamide gel (SDS-PAG). The method involved 1) incorporation of substrate (gelatin or casein) into the SDS-PAG at the time of casting; 2) electrophoresis of the protease inhibitors in the presence of SDS; 3) removal of SDS by washing the gel in 2.5% (w/v) Triton X-100; 4) incubation of the gels in a solution containing the proteolytic enzyme at 37 degrees C for 16 h; and 5) staining undigested substrate with amido black.

Sensitive methods for detecting proteases/protease inhibitors by using fluorescent protease substrates in gels are reported [5,6]. Wilkesman and Schroder [7] used 2-D zymography, a technique that combines IEF (isoelectric focusing) and zymography. Procedures including high-molecular-mass substrates within the gel, such as starch for identification of amylase activity, and protein substrates, such as gelatin, casein, and collagen, for revealing protease/protease inhibitors activity, have been described [8-10].

There are several features of enzyme activity determination including protection of protein functional activity. This provides the possibility of enzyme identification after all biochemical manipulations. Although, it is not possible to save 100% of functional activity, however most of researchers succeeded to save nativity of the protein (including its activity) at the level which is sufficient authentic and adequate identification.

In the present study we report 1D SDS-PAG enzyme electrophoresis method. The technique is optimized for identification of proteases/protease activators of haemostasis system in the blood plasma or other tissue samples.

The major advantage of the method is the possibility to detect active plasminogen activators – tissue-type plasminogen activator (t-PA) and urokinase which simplifies analytical work [11-13]. Enzyme electrophoresis can be used as a rapid diagnostic method as gives information not only about the amount and MW of proteins but also reveals active forms of t-PA and urokinase.

Fibrinogen or (fibrinogen and plasminogen) was incorporated into the gel as a substrate for proteases. The convertion of fibrinogen to fibrin under thrombin treatment provides conditions for fibrin-dependent activators to generate plasmin causing the background substrate degradation [14].

2. MATERIALS AND METHODS

Chemicals and proteins. Tris, glycine, SDS, acrylamide, bisacrylamide, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), sucrose, all of analytical grade, were purchased from Amersham Biosciences AB (Sweden). t-PA, urokinase and elastase were obtained from Sigma, Germany. Streptokinase was supplied by Kabi Pharmacia AG, Sweden.

Sample preparation. Mouse blood plasma from Lewis carcinoma line C57B1/6 was received from Kavetskiy Institute of experimental pathology, oncology and radiobiology of the National Academy of Siences of Ukraine.

Subretinal fluid isolated by surgical operation from patients with regmatogenic retina exfoliation was received from Filatov Institute of eye diseases and tissue therapy of the Academy of Medical Sciences of Ukraine.

The crystalline snake venom of Agkistrodon halys halys was received from serpentarium of Tripolskiy biochemical plant, Ukraine.

All samples were mixed with treatment buffer in the ratio 1:1 (v/v) and stored before electrophoresis at 4˚C. The treatment buffer was made ready according to Amersham Biosciences protocol [15] with modifications: 1) glycerine was replaced by sucrose to the final concentration 5% and 2) DTT was not added to prevent the loss of enzyme activity. The obligatory condition for enzyme electrophoresis samples preparation was non-thermal treatment of the samples before separation to avoid the loss of enzyme activity.

3. ENZYME ELECTROPHORESIS

We have developed a technique on the basis of the method described by Heusen C. and Dowdle E., [16] with following modifications: fibrinogen (1 mg/mL) or (fibrinogen (1 mg/mL) and Glu-plasminogen (10 mkg/mL) was incorporated into separating PAG. Fibrinogen was used to detect proteases capable of fibrinogen cleavage (in this study plasminogen and mini plasminogen, see Example 4, **Figures 4(a) and 5**). Fibrinogen and plasminogen were incorporated into SDS-PAG for plasminogen activators detection. After separation the gel was incubated in thrombin solution (1 NIH/mL) for 1 h at 37˚C. Fibrin formation was required for development of the fibrin-dependent plasminogen activators (t-PA and urokinase) activity [17]. t-PA or urokinase appeared as the clear bands, corresponding to the area where plasmin has degraded fibrin. The separation gel concentration can vary from 11% to 15% to prevent migration of incorporated proteins during electrophoresis.

The technique involved: 1) incorporation of fibrinogen or (fibrinogen and plasminogen) into the SDS-PAG of required concentration; 2) electrophoretic separation under usual conditions [15]; 3) gel washing in 2.5% Triton X-100 with shaking for 1 h at 25˚C for SDS removal; 4) for (fibrinogen and plasminogen)-incorporated gel —treatment with thrombin solution with shaking for 1 h at 37˚C; 5) proteins visualization according to standard protocol [15].

For electoforesis performing and gel staining procedures Hoefer Mighty Small system and Hoefer Processor Plus (Amersham Biosciences AB, Sweden) were used.

The sensitivity of the method was ≤0.01 IU of activator or protease per track.

4. WESTERN BLOTTING

Procedure was performed according to the protocol [17]. Proteins were transferred to a nitrocellulose membrane for 1 h at 4˚C and 60 MA in 15 mM Tris-HCl, pH 8.4 with 120 mM glycine and 20% methanol. The membrane was stained with 0.1% Ponso "Sigma" in 5% acetic acid with shaking for 30 min followed by overnight incubation in 5% BSA at 4˚C. Proteins were probed using monoclonal antibody (MAb) directed against plasminogen (Merck KGaA, Germany) in dilution 1:1000 and secondary antibody (1:3000) labeled with alkaline phosphatase. Each procedure was followed by rinsing step with 3 buffer substitutions. The washing buffer consisted of 50 mM Tris-HCl with 0.1% Twin-20. The blot was developed using 5-bromo-4-chloro-3-indolyl phosphate.

5. PROTEINS PURIFICATION

Human Glu-plasminogen was purified from citrate-anticoagulated blood plasma by affinity chromatography on Lys-Sepharose [18]. The citrate-anticoagulated blood plasma was diluted 1:1 with 50 mM sodium-phosphate, pH 7.4 containing aprotinin (1000 IU/L), filtered and loaded onto Lys-Sepharose column previously equilibrated in the same buffer lacking aprotinin. After loading the column was washed with 50 mM sodium-phosphate, pH 7.4 with 200 mM NaCl, and eluted with 150 mM 6-aminohexacapronic acid in the same buffer. By adding

ammonium sulfate (41 mg/ml), crude ***plasminogen*** was ***precipitated*** from the eluate and gel-filtrated using Sephacryl S-200 column. Purified plasminogen was stored frozen at –20˚C until used.

Activation of plasminogen was performed on a column loaded with insolubilized urokinase. [19]. Glu-plasminogen was incubated within the column for 1 h at 37˚C in 50 mM Tris-HCl, pH 7.4 with 150 mM NaCl and 25% glycerol. The purity of plasmin was controlled by SDS-PAGE and found to be homogenous. The protein was stored frozen at –20˚C in 50% glycerol.

Mini-plasmin was obtained according to the method described by March, Parikh and Cuatrecasas [20]. Glu-plasminogen (10 mg per 1 ml) was hydrolyzed with pancreas elastase 1:50 (v/v) in 50 mM Tris-HCl, pH 8.5 with 100 mM NaCl for 5 h at 25˚C. The reaction was stopped by adding of pNFGB to final concentration 10 mM. The hydrolyzate was loaded onto Superdex 75 for mini-plasminogen separation from cryngls 4 and 1-3. The purified mini-plasminogen was lyophilized and stored at –20˚C. The purity of mini-plasminogen was controlled by SDS-PAGE and found to be homogenous.

6. RESULTS AND DISCUSSION

Human, rabbit, bovine, porcine and mouse haemostasis proteases/protease activators were visualized through this method. To provide optimal results fibrinogen and plasminogen of target mammal should be used for incorporation. However, authors examined usage of human plasminogen and fibrinogen as a background substrate for haemostasis proteases of mammalian species listed above. This significantly simplifies the analysis [21-25].

Advantages and possibilities of enzyme-electrophoresis method are shown in four different cases. In the present study authors did not bring out data of alternative techniques of haemostasis system analysis. Previous reports have confirmed results obtained by enzyme-electrophoresis, references to original studies are indicated in the text and included to the list.

Example 1: The increased risk of acute myocardial infarction (AMI) is associated with reduced fibrinolytic activity. Increased levels of thrombin-antithrombin III complex, fibrinopeptide A, prothrombin fragment F1 + 2 and D-dimer are detectable in patients affected by thrombosis. [26-30]. The level of t-PA antigen is increased but associated with decreased t-PA activity [31-33].

For patients who had AMI, we documented an increased fibrinolytic potential after streptokinase (Sk) administration [21]. To determine the reason of plasmin formation we used rabbit and porcine models of thrombolytic therapy. Whereas Sk activates human and rabbit plasminogen, it fails to activate porcine. This allowed us to distinguish plasmin and Sk effects. The total plasminogen activators were determined using enzyme-electrophoresis. (**Figures 1(a), (b)**). PAG was prepared in the presence of both fibrinogen and Glu-plasminogen. After separation the gel was treated with thrombin. Activity of plasminogen activators was visualized as bands cleared from fibrin by the activated proenzyme included to the gel.

Figure 1(a) demonstrates that rabbit blood plasma contains proteins with fibrinolytic activity and molecular weights of 82, 70 and 54 kDa, which correspond to plasmin, t-PA and two-chain urokinase-type plasminogen activator (tcu-PA). Increment of plasmin and t-PA activities was visible 1 hour after streptokinase administration. Porcine plasma contained the same proteins, but 54 kDa band was barely visible indicating the trace amount of tcu-PA in the sample (**Figure 1(b)**). t-PA activity was significantly increased 1 hour after thrombolytic agent administration. This indicates that t-PA activity is associated with Sk independently of plasmin formation.

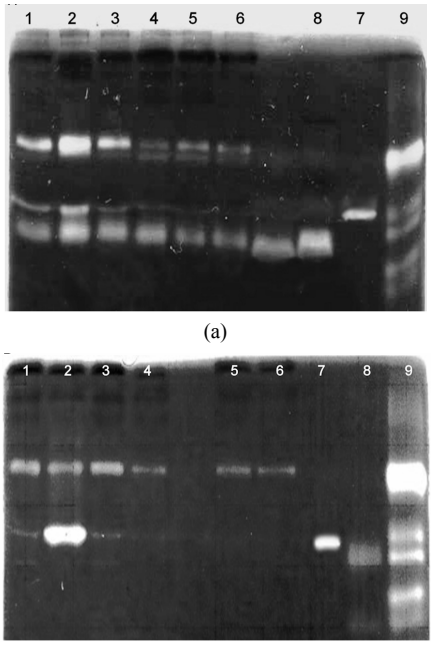


Figure 1. Enzyme electrophoregram of rabbit (a) and porcine (b) blood plasma: 1) control (before streptokinase administration); 2) 1 h after streptokinase administration; 3) 4 h after streptokinase administration; 4) 1 day after streptokinase administration; 5) 3 days after streptokinase administration; 6) 7 days after streptokinase administration; 7) t-PA standard (MW 70 kDa); 8) urokinase standard (MW 56, 33 kDa); 9) plasmin standard (MW 82 kDa).

Example 2: Snake venoms are complex mixtures containing many different biologically active proteins and peptides. A number of these proteins affect the blood coagulation pathway, endothelial cells, and platelets [34]. Several venom enzymes have been used as anticoagulants and other are under examination of their possible therapeutic potential. We used enzyme-electrophoresis to detect potential plasminogen activator in *Agkistrodon halys halys* venom. For this purpose fibrinogen and plasminogen were incorporated into the PAGE. The mixture of previously purified activator and plasminogen 1:1 (q/q) was used as a sample. The gel was not treated with thrombin but after SDS on Triton X-100 substitution was incubated for 12 hours in 50 mM Tris-HCl, pH 7.4 for development of plasmin activity due to action of potential activator. In the analyzed sample (**Figure 2**) two bands were found. One of them corresponded to 82 kDa plasmin, which had been formed under activator action. The second band corresponded to MW of activator itself (32 kDa), its appearance was provided by plasminogen incorporation. These results suggest that induction of plasmin formation by the snake venom activator was specific and involved a bond cleavage at a specific site in the plasminogen molecule without formation of active fragments.

These results were completely confirmed using specific chromogenic substrate for plasmin [22].

Example 3: To detect components of plasminogen activation system in the subretinal fluid of patients with regmatogenic retina exfoliation enzyme-electrophoresis was performed. This substance is accumulated in the cavity formed by pathologic retina exfoliation. It was shown that subretinal fluid possessed fibrinolytic activity and included an activator capable of plasminogen trans formation. To determine the nature of this activator (or activators) fibrinogen and plasminogen-incorporated gel was used. After separation and SDS removal the gel was incubated in thrombin for fibrin formation. This step was necessary due to fibrin-dependent nature of t-PA and urokinase activities. The 54 kDa band was revealed at the electrophoregram (**Figure 3(a)**) and confirmed the existence of tcu-PA in the sample.

Previous reports have confirmed results obtained by substrate-incorporated electrophoresis [23,35].

Example 4: ***Production of elastase*** is significantly enhanced in tumor cells leading to formation of plasminogen internal fragments (angiostatine that consists of kringles 1 to 4 and approximately 85% of kringle 5) [36-39]. Unbound plasminogen/plasmin cringles are biologically active molecules that act on distant sites affecting fibrinolytic efficiency [39-42].

Using enzyme-electrophoresis method we analyzed proteases and plasminogen activators composition in the blood plasma of Lewis carcinoma mice (**Figure 4(a)**). The analysis of haemostasis system during Lewis carcinoma growth is reflected in the study [24].

Fibrinogen and Glu-plasminogen were incorporated into the PAG. After separation of the samples and SDS removal the gel was incubated in thrombin solution for fibrinogen transformation. Appearance of active bands revealed plasminogen activators and proteases capable of fibrin cleavage. As migration pattern of the size (Mr) standards elastase (27, 29 and 31 kDa), urokinase (33 and 56 kDa), tPA (70 kDa) and mini-plasmin (36 kDa) were used. The resulting electropherogram for this case

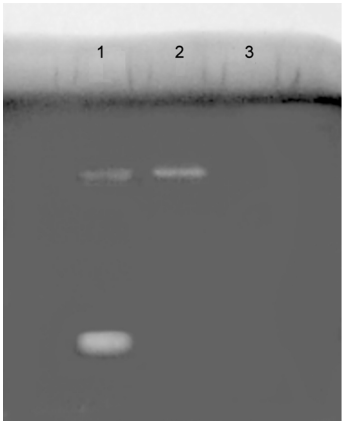


Figure 2. Enzyme electrophoregram of plasminogen and plasminogen activator from snake venom mixture: 1) plasminogen and plasminogen activator mixture; 2) plasmin standard (MW 82 kDa); 3) plasminogen standard used for incubation and incorporation.

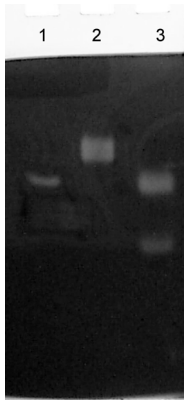
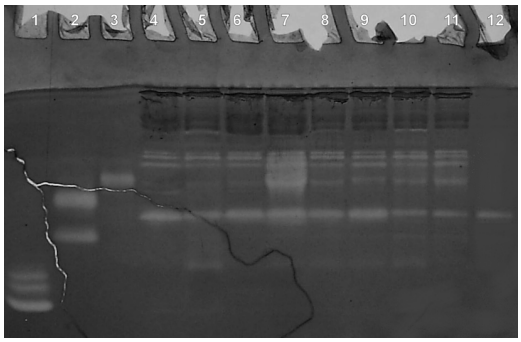
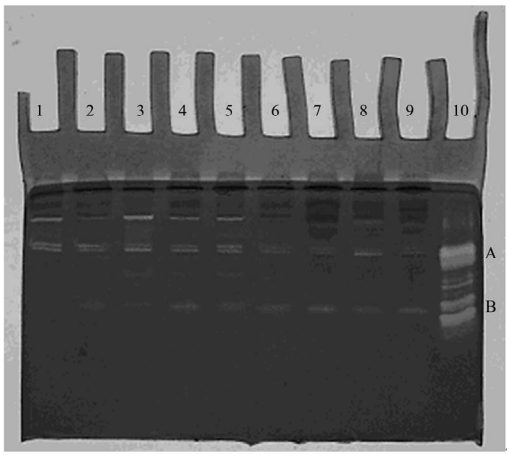


Figure 3. Enzyme electrophoregram of subretinal fluid: 1) subretinal fluid; 2) t-PA standard (MW 70 kDa); 3) urokinase standard (MW 56, 33 kDa).



(a)



(b)

Figure 4. (a) Enzyme electrophoregram of plasminogen activators and active proteases from Lewis carcinoma mice blood plasma: 1) elastase standard (MW 31, 29, 27 kDa); 2) urokinase standard (MW 56, 33 kDa); 3) t-PA standard (MW 70 kDa); 4-11) blood plasma samples; 12) miniplasmin standard (MW 36 kDa); (b) A Enzyme electrophoregram of active proteases capable of fibrinogen cleavage from Lewis carcinoma mice blood plasma: 1) control blood plasma; 2-9) Lewis carcinoma mice blood plasma samples; 10) standards: A-plasmin (MW 82 kDa), B-miniplasmin (MW 36 kDa).

is shown on the **Figure 4**. All samples displayed an active zones with MW of 31 kDa, which corresponded to elastase, 36 kDa (mini-plasmin), 70 kDa (t-PA) and 33 kDa (urokinase). Some samples contained high molecular weigh urokinase (band with MW of 56 kDa).

In order to detect active proteases capable of fibrinogen cleavage, incorporation of plasminogen and fibrinogen into fibrin transformation steps were excluded. This analysis of Lewis carcinoma mice blood plasma revealed active zones with MW corresponding to plasmin (82 kDa) and mini-plasmin (36 kDa). To confirm our results western-blot with MAb directed against plasminogen [43] was performed (**Figure 5**). Data obtained by western blotting testified that enzyme electrophoresis could be used for plasmin and mini-plasmin detection.

Figure 5. Western-blot of Lewis carcinoma mice blood plasma with MAb directed against plasminogen: 1) plasmin standard (MW 82 kDa); 2) miniplasmin standard (MW 36 kDa); 3) blood plasma sample.

7. CONCLUDING REMARKS

Our findings indicate that enzyme-electrophoresis method shows reliable results for identification of active t-PA and urokinase. The technique can be used for studying of protease composition and protein-protein interactions in haemostasis system. The total procedure is quite quick and simple and can be recommended as alternative medical diagnostic method used for the rapid assessment of plasma fybrinolytic potencial.

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