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OPTIMIZATION OF AN ENZYME ELECTROPHORESIS METHOD FOR USING COLLAGEN AS A SUBSTRATE

Background. *The optimized method of collagen-containing sodium dodecyl sulfate-polyacrylamide electrophoresis is introduced in this work. The main parameters that affect the efficiency and resolution of the methodare the degree of sample dilution, the concentration of separation gel,the concentration of collagen solution which was used as a substrate, time of electrophoretic separation elongation. In order to investigate all active and pro-active forms of plasmin, all samples were prepared in two variations: with supplementary activation by streptokinase (Sk) addition, and without.*

Methods. *To obtain clearly visible and detectable lysis points, samples with Sk were diluted in a ratio of 1:16, and samples without Sk were diluted in a ratio of 1:8. To prevent substrate migration and the loss of proteins' electrophoretic mobility, the concentration of was 15 % separation gel, and the concentration of copolymerized collagen solution was 1 mg/ml. To obtain the most informative results, the time of electrophoresis elongation was 15 min. After electrophoresis gels were washed in 2,5 % Triton X-100 solution for 1 hour and stained according to a standard protocol.*

Results. *As a result of the conducted research, the optimal conditions for carrying out this enzyme-electrophoresis modification were found, according to all analytical manipulations, and methodical approaches to the detection of latent pro-enzymatic forms of enzymes with collagenolytic activity were shown.*

Conclusions. *This modified technique can be used for quantitative and qualitative analysis of the presence of collagenolytic activity in various samples and allows research of enzymes that possess this activity, both from a scientific point of view, and in the process of finding and developing technologies for obtaining collagenolytic enzymes for biotechnological purposes.*

Keywords: *enzyme electrophoresis, collagen, collagenolytic activity, proteolytic activity.*

Background

The human genome has more than 500 genes that encode proteases or protease-likemolecules, where the largest classes are metalloproteinases (MMPs) andserineproteases (Kundapur, 2013). These enzymes regulatea lot of biological processes. For example, MMPs are important for tissue repair and remodeling, they also play a crucial role in different pathological processes, so that they were used as diagnostic tools or therapeutic targets in a wide range of diseases. Several approaches for detecting and identifying proteolytic activity in various samples have been developed, but enzyme electrophoresis remains to be the preferred one due to its simplicity and quality (Choi et al., 2008; Tajhya et al.,2017).

Enzyme electrophoresis is based on a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique with an incorporated substrate thatallows scientists to detect active enzymesby substrate degradation (Wilkesman, 2009). This method has a lot of advantages, for example, this technique gives both quantitative and qualitative information, it allows not only to detect the presence of different enzymes but alsoto identify them by molecular weight. In addition, both pro-enzymatic and active forms can be distinguished by zymography (Snoek-van Beurden, & Von den Hoff, 2005). However, the main advantage of this method is that it can be developed for the detection of different proteases by incorporating a suitable substrate such as fibrinogen, gelatin, casein, and collagen (Choi et al., 2004).

The inclusion of native collagen fibers in PAAG seemed to be in appropriate, duetoits complex structure. However, this problem was solved by SDS presence. SDS disruptsmost of the fibrillar collagen organization, which allows proteins to easily migrate through the gel and partiallyp reserve their electrophoretic mobility (Gogly et al.,1998). The first data indicating the successful identification of active collagenase were obtained by Gogly and colleagues (Gogly et al.,1998)in their study on the use of copolymerized native collagen I in SDS-PAGE for the determination of interstitial collagenase. Although collagen zymography had been proven to be more sensitive than gelatin zymography because it can detect up to 0.1 pg of active collagenase (MMP-1) (Gogly et al.,1998), gelatin is still a commonly used substrate for protease detection and the information of collagen usage is limited.

In this study, we present an optimized method of collagen enzyme electrophoresis for qualitative and quantitative detection of collagenolytic activity in samples. The main advantages of this technique are that it prevents substrate migration through the gel and provides a better level of washing and staining steps for gaining clearly detectable results.

Methods

Tris, acrylamide, N,N'-methylen-bis-acrylamide, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), Triton X-100, glycine, Coomassie G-250, were purchased from GE Healthcare AB, Sweden. Streptokinase was obtained fromKabi Pharmacia AG, Sweden.

To test the technique we used blood plasmafrom donors, who had recovered from COVID-19. According to the available scientific literature (Rachkovska et al., 2023), the post-COVID period is characterized by hemostatic

disorders, which can lead to an increase in proteolytic activity in the blood stream.

Samples were prepared in two variations, according to a protocol (Bratchik, 1993): 1) blood plasma was diluted in distilled water in a ratio of 1:10 without streptokinase (Sk) addition in order to investigate the whole content of active plasminogen/plasmin forms; 2) blood plasma was incubated with Sk for 10 min at 37 °C before dilution to examine pro-enzymatic forms with plasminogen origin present in the bloodstream. Then, all samples were incubated with 0,25 % acetic acid for 30 min at 4 °C and centrifugated for 15 min at 10,000 g. The precipitate was mixedwith a sample buffer in an appropriate volume and stored before electrophoresis at 4 °C without boiling or the addition of any reducing agent, in order to prevent the loss of enzymatic activity.

Collagen zymography was conducted in several steps. A separation gel with collagen was incubated for 1 hour at room temperature in order to gain an appropriate polymerization. Electrophoresis was carried out at 36 mA for concentration gel and at 72 mA for separation gel. Then the gel was incubated with a non-ionic detergent, 2.5 % Triton X-100 solution for 1 h with gentle orbital shaking toremove the SDS.This step is necessary for enzyme and substraterenaturation, it also helps to prevent the irreversible denaturation of some enzymes and the total loss of their activity.Subsequently, gelswere incubated in Tris-buffered saline solution with pH 7.4 for 12 h at 37 °C, in order to activate the enzyme and ensure that the substrate was hydrolyzed at its stopping points in the gel. Afterthe obtained gel was stained with Coomassie Brilliant Blue and washed to detect lysis spots. The results were detected as clear lysis points on a dark background. As markers of molecular weight were used trypsin (23 kDa) and plasmin (85, 44, 36 kDa). Plasmin was prepared according to a standard protocol (Wiman, 1973).

Results

We determined the main parameters that can affect the quality of electrophoresis: 1) degree of sample dilution; 2) concentration of separation gel; 3) concentration of collagen solution which was added to the separation gel as a substrate; 4) time of electrophoretic separation elongation after sample front line extends beyond gel into a buffer for electrophoresis.

Testing samples without dilution can lead to excessive hydrolysis of the substrate, making the results difficult to analyze. However, the greater the sample dilution, the smaller the lysis points. The optimum degree of sample dilution was investigated by placing identical samples with and without Sk on different gels with dilutions of 4, 8, and 16 times. Samples with Sk diluted 16 times (Fig. 1a), compared to the others, formed a larger number of bands evenly distributed across the gel (it is shown by arrows that point to the lysis areas). On the opposite, samples without Sk diluted 8 times (Fig. 1b) allowed us to detect a larger number of clearly visible bands (it is shown by arrows that point to the lysis areas).

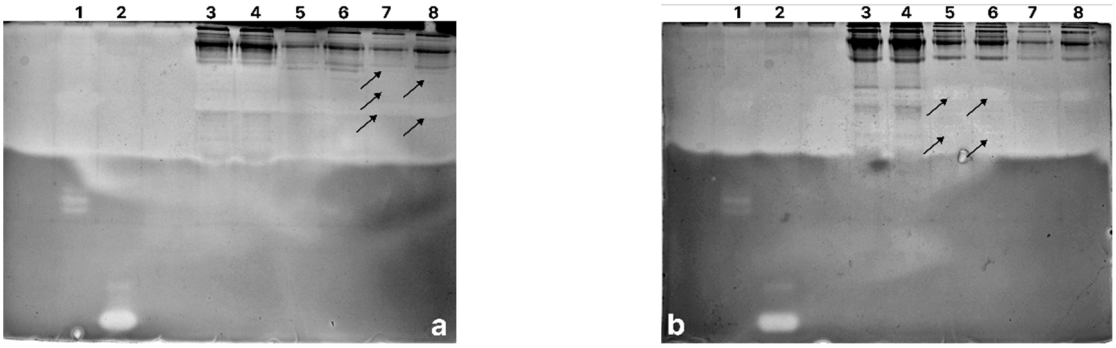

Fig. 1. Enzyme electrophoregram of donors'blood plasma incubated with Sk (a), and with out additional incubation withSk (b): 1 – plasminstandard (MW85, 44, 36 kDa); 2 – trypsin standard (MW23 kDa); 3, 4 – samplesdiluted 4 times, with 0 Index (S/C) titer of anti-SARS-CoV-2 IgG; 5, 6 – samples diluted 8 times, with 0 Index (S/C) titer of anti-SARS-CoV-2 IgG; 7, 8 – samples diluted 16 times, with 0 Index (S/C) titer of anti-SARS-CoV-2 IgG

The concentration of separation gel,due to pore size, has a great impact on a protein's electrophoretic mobility, substrate incorporation, and its ability to migrate through the gel. We revealed that the 10 % separation gel caused substrate migration and neither m. w. markers nor samples were not detectable (Fig. 2, the arrow points to the line of collagen migration).

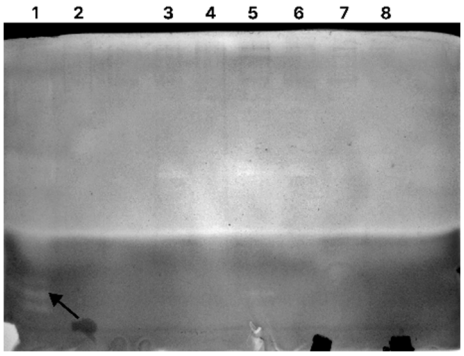

Fig. 2. Enzyme electrophoregram of donors' blood plasma incubated with Sk, separated in 10 % gel: 1 – plasmin standard (MW 85, 44, 36 kDa); 2 – trypsin standard (MW 23 kDa); 3, 4, 5 – sample diluted 16 times, with 10 ± 3 Index (S/C) titer of anti-SARS-CoV-2 IgG; 6, 7, 8 – sample diluted 16 times, with 55 ± 5 Index (S/C) titer of anti-SARS-CoV-2 IgG

On the opposite, 12 % gel prevented the loss of samples and allowed to get distinct lysis points, but it failed with substrate migration (Fig. 3, the arrow in the middle points to the line of collagen migration).

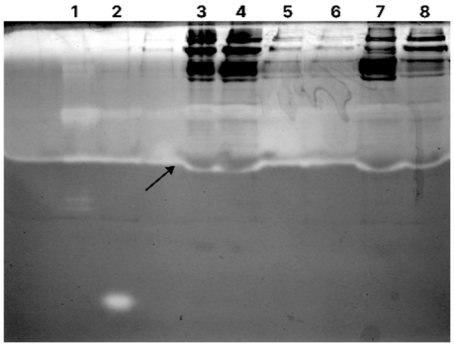

**Fig. 3. Enzyme electrophoregram of donors' blood plasma incubated withSk, separatedin 12 % gel:
1 – plasmin standard (MW 85, 44, 36 kDa); 2 – trypsin standard (MW 23 kDa); 3, 4, 5 – samples diluted 16 times, with 0 Index (S/C) titer of anti-SARS-CoV-2 IgG; 6, 7, 8 – samples diluted 16 times, with 10 ± 3 Index (S/C) titer of anti-SARS-CoV-2 IgG**

Fig. 4 shows that the 15 % separation gel contributes to keeping collagen from migrating out of the gel andthe formation of evenly distributed, clearly visible markers and samples (the square on the gel shows the line of lysis points).

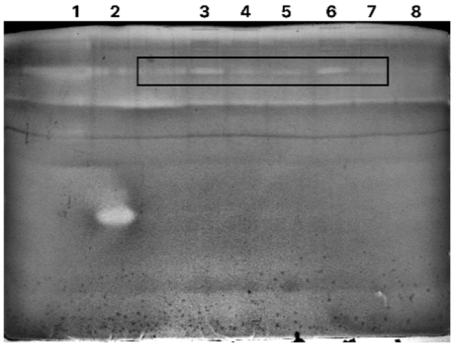

**Fig. 4. Enzyme electrophoregram of donors' blood plasma incubated with Sk, separated in 15 % gel:
1 – plasmin standard (MW 85, 44, 36 kDa); 2 – trypsin standard (MW 23 kDa); 3, 4, 5 – sample diluted 16 times, with 10 ± 3 Index (S/C) titer of anti-SARS-CoV-2 IgG; 6, 7, 8 – sample diluted 16 times, with 55 ± 5 Index (S/C) titer of anti-SARS-CoV-2 IgG**

We determined that 18 % gel, shown in Fig. 5 has a critically low pore size, so that samples weren't able to normally migrate and separate through the gel (the square on the gel shows the front of samples and marker).

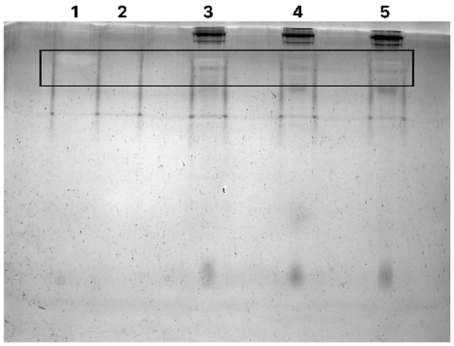

**Fig. 5. Enzyme electrophoregram of donors' blood plasma incubated with Sk, separated in 18 % gel:
1 – plasmin standard (MW 85, 44, 36 kDa); 2 – trypsin standard (MW 23 kDa);
3, 4, 5 – samples diluted 16 times, with 0 Index (S/C) titer of anti-SARS-CoV-2 IgG**

The concentration of substrate copolymerized in to the separation gel can affect the protein'sability to migrate through the gel and the quality of washing gel after staining. It was found that a 2 mg/ml collagen solution cause dpoor washing of the gel after staining, which made both markers and samples badly visible (Fig. 6a, the arrows point to the markers). In contrast, the zymogram in Fig. 6b shows that the collagen solution with a concentration of 1 mg/ml contributed to better washing after staining, which allowed us to obtain clearly visible molecular weight markers and samples (the arrows on the gel also point to the markers).

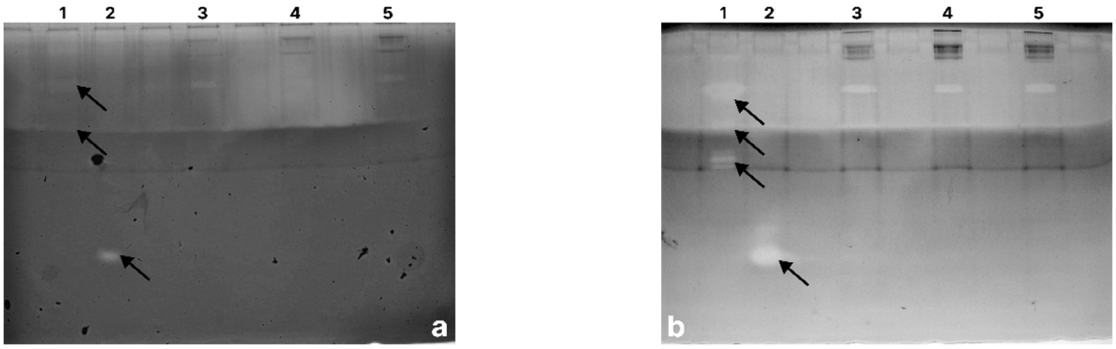

Fig. 6. Enzyme electrophoregram of donors' blood plasma in cubated with Sk, separated in 15 % gel with in corporated 2 mg/ml (a), and 1 mg/ml (b) collagensolution:1 – plasmin standard (MW 85, 44, 36 kDa); 2 – trypsin standard (MW 23 kDa); 3, 4, 5 – samples diluted 16 times, with 0 Index (S/C) titer of anti-SARS-CoV-2 IgG

Despite the fact that substrate incorporation practically does not create additional obstacles to the protein's movement through the gel, their electrophoretic mobility is still reduced to some extent, which can be detected by m. w. markers. So thetime of electrophoretic separation needs to be elongated after the sample front line extends beyond the gel into the buffer for electrophoresis.

The extension time in 10 minresulted in sample concentration in the up per part of the gel, and the frontline of the markers was higher than expected (Fig. 7a, the square on the gel shows samples' concentration in the upper part of the gel). In contrast, Fig. 7b indicates that extending the electrophoretic separation by 15 min contributes to the formation of a clearer and more elongated exit line for both markers and samples, which caused formation a greater number of bands (the arrows on the gel point to the lysis points distributed across the gel).

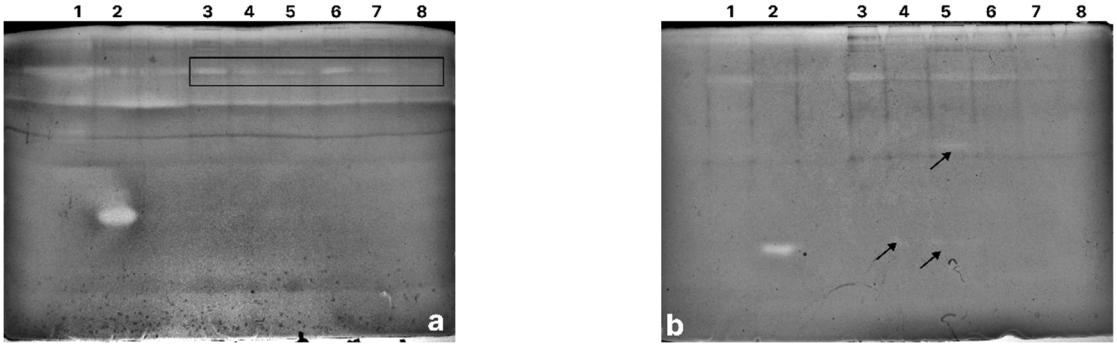

Fig. 7. Enzyme electrophoregram of donors' blood plasma incubated with Sk, with time of electrophoretic separation elongation 10 min (a), and 15 min (b): 1 – plasmin standard (MW 85, 44, 36 kDa); 2 – trypsin standard (MW 23 kDa); 3, 4, 5 – sample diluted 16 times, with 10 ± 3 Index (S/C) titer of anti-SARS-CoV-2 IgG; 6, 7, 8 – sample diluted 16 times, with 55 ± 5 Index (S/C) titer of anti-SARS-CoV-2 IgG

Discussion and conclusions

We found the most appropriate conditions for conducting enzyme-electrophoresis using collagen as a substrate. This optimized technique can be used to test the presence of collagenolytic activity, identify enzymes due to their molecular weight, and compare enzymatic activity in different samples.

Authors' contribution: Maryna Kalashnikova – conducting the experiment and calculations, writing the article; Olexiy Savchuk – conducting the experiment and calculations; Vitaliy Karbovskiy – the idea and conceptual provisions of the conducted research, writing the article.

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ОПТИМІЗАЦІЯ МЕТОДУ ЕНЗИМ-ЕЛЕКТРОФОРЕЗУ ДЛЯ ВИКОРИСТАННЯ ЯК СУБСТРАТ КОЛАГЕНУ

В с т у п . ***Представлено оптимізований метод ензим-електрофорезу з використанням колагену як субстрат. Основними параметрами, що впливають на ефективність та роздільну здатність методу, є ступінь розведення проб, концентрація розподільчого гелю, концентрація розчину колагену, кополімеризованого в розподільчий гель та час продовження електрофоретичного розділення.***

М е т о д и . ***Для того, щоб дослідити всі активні та проактивні форми плазміну, усі зразки були приготовлені у двох варіаціях: з додатковою активацією, шляхом додавання стрептокінази (Ск), та без неї. Для отримання чітко видимих точок лізису зразки із Ск розводили у співвідношенні 1:16, а зразки без Ск розводили у співвідношенні 1:8. Для запобігання міграції субстрату та втрати електрофоретичної рухливості білків використовували розподільчий гель з концентрацією 15 %, а концентрація розчину колагену, кополімеризованого в гель, становила 1 мг/мл. Для отримання найбільш інформативних результатів час продовження електрофоретичного розділення становив 15 хв. Після електрофорезу гелі відмивали в 2,5 % розчині Тритон Х-100 протягом 1 год та фарбували відповідно до стандартного протоколу.***

Р е з у л ь т а т и . ***У результаті проведенних досліджень було знайдено оптимальні умови проведення модифікації ензим-електрофорезу згідно з усіма аналітичними маніпуляціями та показано методичні підходи до виявлення латентних профементних форм ензимів, яким властива колагенолітична активність.***

В и с н о в к и . ***Описану модифіковану методику можна використовувати для кількісного та якісного аналізу наявності колагенолітичної активності в різних зразках, що дозволяє проводити дослідження ферментів, яким властива така активність, як з наукових позицій, так і в процесі пошуку та розроблення технологій отримання ферментів-колагенолітиків для біотехнологічних цілей.***

К л ю ч о в і с л о в а : ***ензим-електрофорез, колаген, колагенолітична активність, протеолітична активність.***

Автори заявляють про відсутність конфлікту інтересів. Спонсори не брали участі в розробленні дослідження; у зборі, аналізі чи інтерпретації даних; у написанні рукопису; в рішенні про публікацію результатів.

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; in the decision to publish the results.