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Short Communication

Chip gel electrophoresis as a tool for study of matrix metalloproteinase 9 interaction with metallothionein

Matrix metalloproteinases (MMPs) play numerous roles in physiological and pathological processes including cancer. Interactions of MMPs with other zinc-binding proteins are of great interest mainly from the point of view of the participation of these proteins in carcinogenesis. The aim of this study was to utilize chip gel electrophoresis for investigation of matrix metalloproteinase 9 (gelatinase B, MMP-9) interactions with collagen. The interaction was observed and the effect of temperature was tested. We further focused on the study of activation of MMP-9 by the presence of zinc-binding proteins called metallothioneins (MT). We confirmed the basic presumption of the activation of MMP-9 by the presence of MT similar to the temperature effect. Moreover, we chose the method of differential pulse voltammetry Brdicka reaction to verify our results. It can be concluded that the activity of MMP-9 was higher in the presence of MT in comparison with the experimental collagen(type I)-MMP-9 mixture in vitro.

Keywords:
Chip gel electrophoresis / Interaction / Matrix metalloproteinases / Metallothionein / Voltammetry
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Matrix metalloproteinases (MMPs) were discovered in 1962 [1]. Approximately, 20 types of MMPs have been identified and are labelled with numbers ranging from MMP-1 to MMP-28 [2]. The best-known physiological role these proteins play is cleaving and rebuilding connective tissues, such as collagen and elastin [3]. MMPs also participate in angiogenesis [4]. MMPs, furthermore, play a role in a number of pathological processes such as arthritis, Alzheimer's disease, atherosclerosis, vascular disease, gastritis ulcer disease, central nervous system disease, liver cirrhosis and pro-angiogenic activities in malignant tumours [5–7]. MMPs can be used as markers for some cancers including colorectal, thyroid, bladder and breast cancer, neurodegenerative, immune and cardiovascular diseases [8]. Assays for the detection of MMPs for both clinical and research purposes are summarized in the following reviews [9–13].

Literature surveys show great interest in studying the biological functions of MMPs; however, methodological heterogeneity makes it difficult to compare the results between studies and to draw conclusions from the results. Lack of analytical validation methods is one of the challenges faced in MMP detection in clinical research. Enzymatic, immunochemical, fluorimetric in vivo imaging methods are commonly used techniques in clinical research [7]. Immunochemical methods precede enzymatic methods, but cannot distinguish between active MMP and inactive MMP in zymogene form [9]. Fluorimetric methods using fluorescent-labelled substrates show low detection limits but allow to determine MMP activity quantitatively and to study target MMP sequences [10]. There are a number of other methods that are subjects of interest such as phage display, Multiple-Enzyme/Multiple-Reagent Assay System (MEMRAS) and activity-based profiling including reversed zymography.

The aim of this study is to utilize chip gel electrophoresis for investigation of matrix metalloproteinase 9 (gelatinase B, MMP-9, Fig. 1) interactions with collagen (type-I). The technique is further used to confirm a possibility of activation of the enzyme by the presence of metallothioneins (MT). These proteins belong to the group of intracellular and low-molecular-mass proteins (from 5 to 7 kDa) in humans. MTs are also considered to play important; however, no clear role in carcinogenesis exists [14–16]. The interactions between various types of MMP and MT have been studied, but the results remain controversial [17–20].

Primarily, chip gel electrophoresis was utilized for studying the interactions between MMP-9 and bovine

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Abbreviations: MMPs, matrix metalloproteinases**; MT,** metallothioneins

Colour Online: See the article online to view Figs. 1-3 in colour.

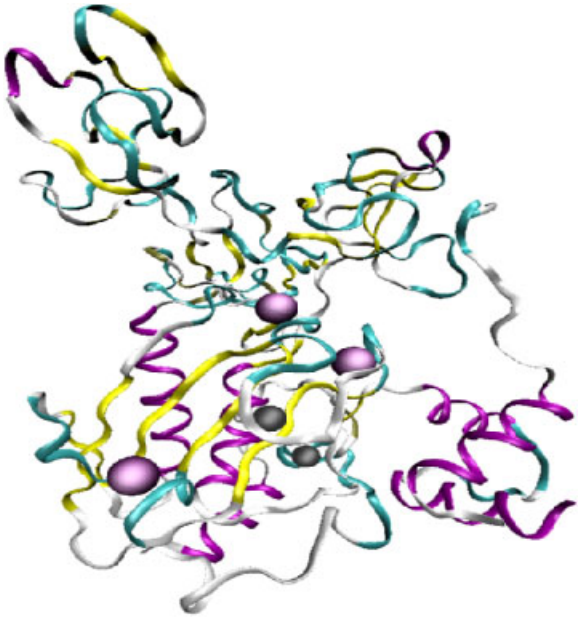

Figure 1. Structure of MMP-9 with Zn(II) (Grey) and Ca(II) (violet) atoms. Source: www.expasy.org.

collagen (type-I). The measurements were carried with Experion. Particularly, we used a plastic chip designed for protein analysis (Pro 260®). There are ten individual wells for an application of samples and a place for the application of a standard mixture of proteins (Ladder 260®) in the chip. The system buffer is first applied. Then, the chip is inserted into the device, which fills the capillaries by this buffer. The application of the samples is as follows. The samples are prepared according to the standard protocol indicated by the manufacturer prior to injecting into wells on the chip. The separation is based on the standard capillary electrophoretic system, where the buffer, which is located in the capillary chip, has the character of the gel. There are also electrodes in the chip, which are connected via capillary tube at the bottom of the chip. A measurement of a sample is rapid lasting app. 1 min depending on the type of a sample and target molecules. The device determines the molecular weight of two standard proteins (ladder), the first one is determined at the beginning and the second at the end of the measurement. Based on this, molecular weights of the target molecules are calculated. The ladder has its own well on a chip. This well is always analyzed as the first one. All target molecules are detected with a fluorescent detector.

According to the manufacturers' protocol, a sample for detection of proteins on a chip Pro 260® is prepared as follows. Each sample was diluted with water to the same protein concentration, 4 μL aliquots was then mixed with 2 μL of reducing sample buffer. After 4 min of boiling (100°C), the sample was cooled and 84 μL of water was added. After priming of the chip with gel and gel-staining solution in the diluted priming station sample, the mixture (6 μL) was loaded into the sample wells. The Pro260® Ladder included in the kit was used as a standard.

Bovine collagen (type-I) (5, 7.5, 10, 15, 20 and 30 ng) was detected for the calibration of measurements. It clearly follows from the results obtained that the height of a standard protein peak decreased with increasing concentration of collagen (Fig. 2A). The decline is characterized by a logarithmic dependence shown in inset in Fig. 2A. Furthermore, we were interested whether MMP-9 would be able to cleave collagen in vitro. We decided to test the activation of the enzyme by increasing the temperature. Two experimental variants were tested. They differed in the amount of added MMP-9 only. In the first one, the mixture of 100 ng of MMP-9 and 20 ng of collagen was used. The second differed in the higher quantity of MMP-9 up to 50 ng only, collagen content remained unchanged. The amount of substances have been chosen with regard to the fact that the larger amount of collagen resulted in the marked lowering of the height of standard protein peak, which also shows the calibration of this protein (Fig. 2A). Incubation (30 min) of samples was carried out at 25, 40 and 65°C. In both concentration variants, we observed a similar trend of increasing and decreasing peak heights of the standard protein. The effect of temperature on cleaving 20 ng of collagen with 100 ng MMP-9 is shown in Fig. 2B. The difference in heights of signals measured in the second variant was similar to the first one. It follows from the results obtained that the activity of MMP-9 enhanced with increasing temperature. All measurements can be compared with the collagen control value at 20 ng as the red curve (Fig. 2B). The results obtained on the device Experion were confirmed by experiments carried out by means of vertical sodium dodecyl sulphate polyacrylamide gel electrophoresis (not shown).

We further focused on the study of activation of MMP-9 by the presence of zinc-binding proteins called MT. We confirmed the basic presumption of the activation of MMP-9 by the presence of MT similar to the temperature. Moreover, we were interested whether we would be able to study such interactions by an electrochemical method. For this purpose, we chose the method of differential pulse voltammetry Brdicka reaction (DPV Brdicka reaction) [21]. The following stock solutions were used for measurements: 1 mg bovine collagen (type-I) per mL (in the presence of 9% HCl [22]), MMP-9 10 μg/mL (ACS water), MT 10 μg/mL (ACS water). Primarily, Brdicka supporting electrolyte (1980 μL; 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6)) was pipetted into the electrochemical vessel. Then, collagen (20 μl) was injected into the Brdicka supporting electrolyte. The resulting concentration of collagen was 10 μg/mL. The working solution temperature was increased from room temperature to physiological, i.e. 37°C using a refrigerated circulator Julabo F12 (Germany), after the addition of collagen. Then, three various mixtures were prepared and measured by the DPV Brdicka reaction (Fig. 3A). The first mixture consisted of 20 μg of collagen only (Fig. 3B, blue curve). The second one consisted of collagen (20 μg) with 200 ng of MMP-9 (Fig. 3B, green curve). The third one consisted of collagen (20 μg), MMP-9 (200 ng) and MT (200 ng) (Fig. 3B, red curve). All mixtures were measured for

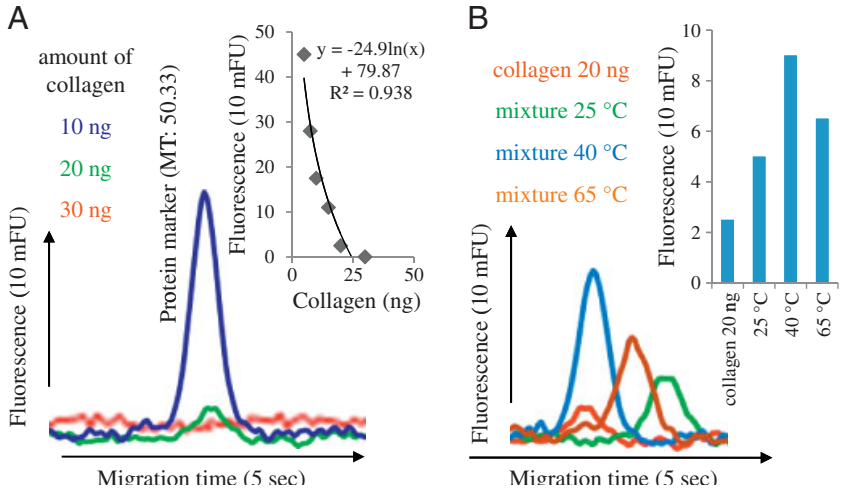

Figure 2. Records obtained from the Experion. (A) Increasing amount of bovine collagen (type-I) simultaneously decreases the height of the standard protein peak, inset: calibration curve. (B) Records obtained for a mixture of collagen (20 ng) and MMP (100 ng) incubated for 30 min at different temperatures before standard sample preparation procedures, inset: the determined peak heights. Measurements were carried out on an automated microfluidic Experion electrophoresis system (Bio-Rad, USA). Human MMP-9 was purchased from the Chemicon International (Temecula, USA). Bovine collagen (type-I) was supplied from the Vyzkumny ustav pletarsky (Brno, Czech Republic). Other chemicals were purchased from Sigma-Aldrich (USA).

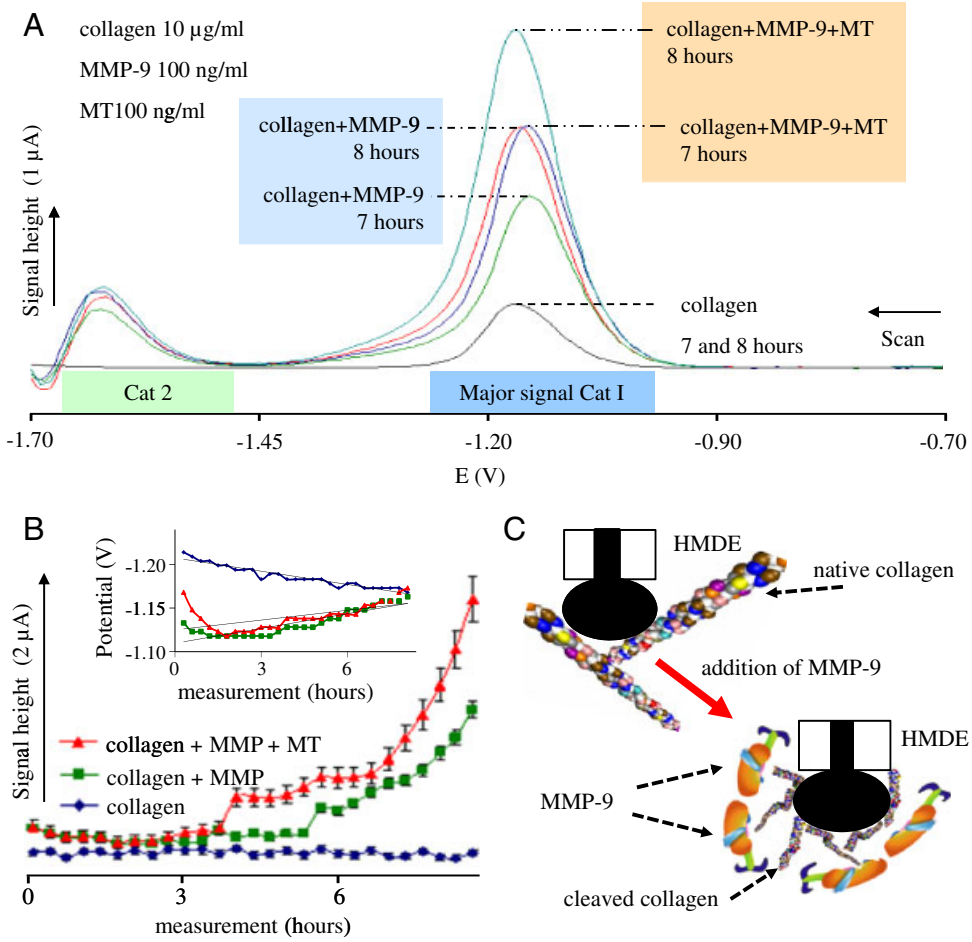

Figure 3. Electrochemical analysis. (A) Typical DP voltammograms of mixtures collagen-MMP-9 and collagen-MMP-9-Zn(II) after 7 and/or 8 h-long incubation. (B) Changes in Cat1 peak height with increasing time of incubation of three mixtures: (i) collagen (20 µg), (ii) collagen (20 µg) and MMP-9 (200 ng) and (iii) collagen (20 µg), MMP-9 (200 ng) and MT (200 ng). Inset: change in the potential of Cat1 peak is characterized by R^2 (Collagen $R^2 = 0.922$, Collagen+MMP $R^2 = 0.771$, Collagen+MMP+MT $R^2 = 0.348$). (C) A scheme of interaction of MMP-9 and MT at the electrode surface. Electrochemical measurements were performed with AUTOLAB Analyzer (EcoChemie, The Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was employed as the working electrode; Ag/AgCl/3 M KCl electrode as the reference electrode; glassy carbon electrode as the auxiliary electrode. For smoothing and baseline corrections, the software GPES 4.9 supplied by EcoChemie was employed. The temperature of the supporting electrolyte was maintained by the flow electrochemical cell coupled with thermostat JULABO F12/ED (Labortechnik, Germany).

more than 8 h in 17 min intervals. During the measurement, solutions were covered with parafilm in an electrochemical cell to prevent evaporation of samples. The tested loss in volume was less than 5% after 10 h. Interaction of collagen with MMP-9 has brought interesting results. Peak, which is responsible for the accessibility of electroactive protein moieties (–SH, $–NH_2$) called Cat1, gradually increased during the incubation of these two proteins. This may be explained by interaction with MMP-9 collagen, in which there was a gradual degradation of collagen and thus increased the number of electroactive groups capable of electrochemical conversion at the surface of working electrode (Fig. 3A and B, green curve). Signal of collagen itself did not change during incubation (Fig. 3B, blue curve). The changes in MMP-9 activity after addition of MT are shown in Fig. 3B, as the red curve. Cat1 signal increased throughout the incubation period. In the 360th minute, the breakthrough occurred and the signal increased over the next several dozen minutes for more than 50% compared to the previous values. The structure of collagen is very complex and it is very likely that breakthrough in the Cat1 peak height indicates cleaving the largest structures of the collagen and then degradation of smaller fragments occurs. The most interesting result is that the activity of MMP-9 is higher in the presence of MT in comparison with the experimental collagen-MMP-9 mixture (Fig. 3B); however, the potential of Cat1 peak did not change during the incubation (inset in Fig. 3B), which shows the fact that protein complexes or higher protein structures did not form during the experiment. Based on our results, we decided to suggest a scheme of interaction of MMP-9 and MT at the electrode surface (Fig. 3C). Zinc(II) ions present in the MT molecule activated MMP-9, which then cleaved collagen. The presence of smaller fragments of collagen, which is characterized by the presence of more free groups able to provide the catalytic signal, increases the peak Cat1.

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The authors have declared no conflict of interest.

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