Full Paper

Chronopotentiometric Stripping Analysis of Gelatinase B, Collagen and Their Interaction

Dalibor Huska,[a] *Vojtech Adam*,[a,b] *Ondrej Zitka*,[a,c] *Jiri Kukacka*,[d] *Richard Prusa*,[d] *Rene Kizek*[a]*

[a] Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic
*e-mail: kizek@sci.muni.cz
[b] Department of Animal Nutrition and Forage Production, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic
[c] Department of Biochemistry, Faculty of Science, Masaryk University, Kotlarska 2, CZ-611 37 Brno, Czech Republic
[d] Department of Clinical Biochemistry and Pathobiochemistry, 2nd Faculty of Medicine, Charles University, V Uvalu 84, CZ-150 06 Prague 5, Czech Republic

Received: July 23, 2008
Accepted: October 12, 2008

Abstract
Matrix metalloproteinases (MMP) belong to a group of zinc-dependent proteins that play a central role in the breakdown of extracellular matrices. Collagen, elastin, gelatin and casein are the main components of extracellular matrix cleaved by MMP. This paper aims to analyze the interaction between gelatinase B (MMP-9) and collagen using chronopotentiometric stripping analysis with adsorptive transfer stripping technique (AdTS CPSA). Under optimized experimental conditions (time accumulation of 90 s, supporting electrolyte 0.2 M acetate buffer pH 5, stripping current 1 μA), the detection limit (3 signal/noise) for MMP-9 was estimated as being 100 pM. The interaction between MMP-9 and collagen was studied according to the following scheme: i) HMDE surface was renewed. ii) Renewed surface of HMDE collagen (1 μg/mL) was accumulated for 90 s under open circuit. iii) The electrode was rinsed in ACS grade water and immersed in 5 μL drop of MMP-9. iv) The interaction between MMP-9 with collagen took place at open circuit. v) The electrode was then rinsed in ACS grade water. vi) The rinsed electrode was transferred into an electrochemical cell and measured in acetate buffer (pH 5). The CPSA signal of collagen after its interaction with MMP-9 increased more than 30% compared to that of only collagen. This increase in signal is likely due to the cleavage of collagen by MMP-9, hence its easy access to the electrode's surface.

Keywords: Matrix metalloproteinases, Chronopotentiometric stripping analysis, Collagen, Protein – protein interaction, Cancer

DOI: 10.1002/elan.200804440

Dedicated to Professor Joseph Wang, on the Occasion of His 60th Birthday

1. Introduction

The matrix metalloproteinases (MMP), also known as matrixins, belong to a group of zinc-dependent proteins, which are thought to play a central role in the breakdown of extracellular matrix. Collagen, elastin, gelatin, and casein are the main components cleaved by MMP. The breakdown of these components is essential for many physiological processes such as embryonic development, morphogenesis, reproduction, and tissue resorption and remodeling [1]. MMP also participate in pathological processes such as arthritis, cancer, cardiovascular and neurological diseases [2 – 6]. The primary structure of MMP, for twenty different vertebrates, is comprised of several domain motifs, as illustrated in Figure 1. The domains have been divided according to their structure and function: collagenases, stromelysins, matrilysins, gelatinases, membrane type MMP and others MMP [7, 8].

Chronopotentiometric stripping analysis (CPSA) measures the evolution of hydrogen from the supporting electrolyte catalyzed by the presence of a protein. This method is a highly sensitive technique commonly used for the analysis of proteins with detection limits at subnanomolar and lower levels. Disadvantages include high standard deviations and time of analysis at high stripping currents [9]. CPSA has been used for the detection of several biologically important peptides [10, 11] and proteins such as metallothionein [12 – 18], α-synuclein protein [19], MutS protein [20], glutathione-S-transferase [21], thrombin [22]. Moreover, Ostatna et al. showed this electrochemical method can be employed to study structural changes of bovine serum albumin [23, 24]. Serrano et al. studied metal – protein interactions using CPSA [25, 26]. Redox states of peptides and proteins can also be determined using CPSA [27]. However, CPSA has not been utilized for the detection of MMP, yet. The main aim of this paper is to characterize MMP-9, collagen and

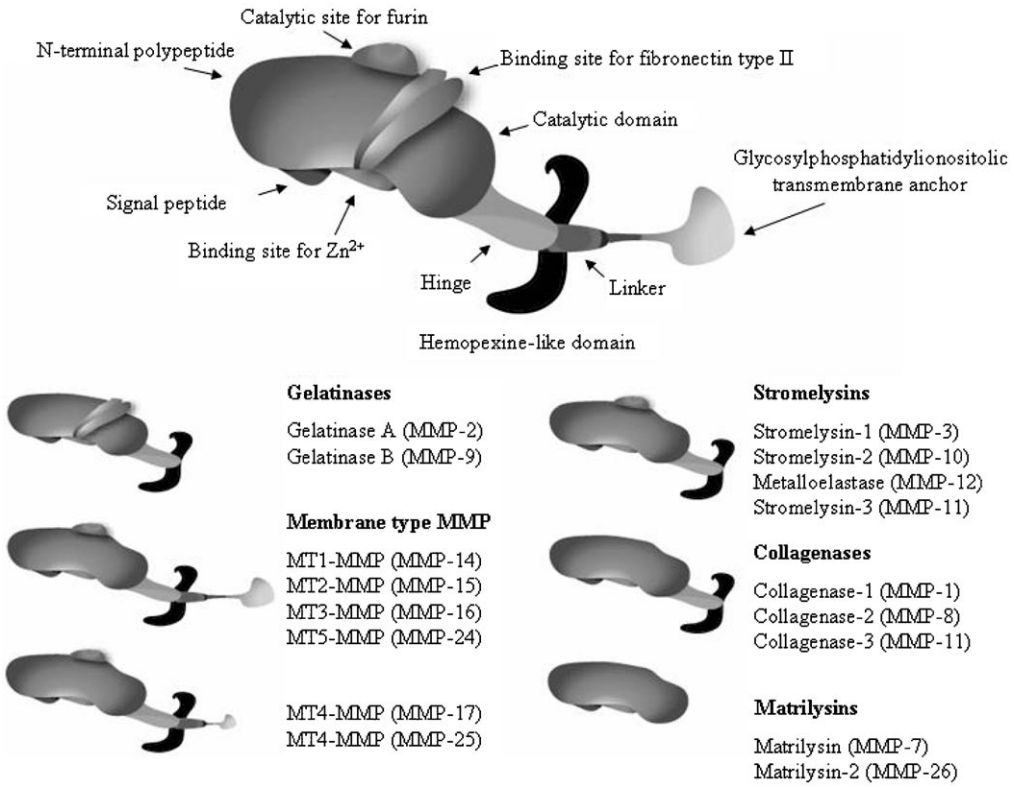

Fig. 1. Characterization of single MMPs according to their structural differences.

their interaction by using chronopotentiometric stripping analysis with adsorptive transfer stripping technique.

2. Experimental

2.1. Chemicals and pH Measurements

Human MMP-9 was purchased from Chemicon International (Temecula, USA). Collagen was supplied from Vyzkumny ustav pletarsky (Brno, Czech Republic). ACS grade $Co(NH_3)_6Cl_3$ and other chemicals (chemicals meet the specifications of the American Chemical Society) used were purchased from Sigma Aldrich (Sigma-Aldrich, USA) unless noted otherwise. The stock standard solutions (10 μg/mL) were prepared with ACS water (Sigma-Aldrich, USA) and stored in the dark at $-20\,^{\circ}C$. Working standard solutions were prepared daily by the dilution of the stock solutions with ACS certified water. The pH and conductivity were measured using inoLab Level 3 (Wissenschaftlich-Technische WerkstÄtten GmbH; Weilheim, Germany).

2.2. Electrochemical Measurements

Electrochemical measurements were performed with AUTOLAB Analyzer (EcoChemie, 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. An Ag/AgCl/3 M KCl electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. For smoothing and baseline corrections, the software GPES 4.9 supplied by EcoChemie was employed. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%) and saturated with water for 120 s. All experiments were carried out at room temperature. The temperature of supporting electrolyte was maintained by the flow electrochemical cell coupled with thermostat JULABO F12/ED (Labortechnik GmbH, Germany).

Adsorptive transfer stripping technique (AdTS) with chronopotentiometric stripping analysis (CPSA) was used to determine the presence of MMP-9 and/or collagen by recording the inverted time derivation of potential $(dE/dt)^{-1}$ as a function of potential E [12]. Peptides and proteins produce a well-developed peak at highly negative potentials [10]. The behavior of this peak suggests the presence of catalytic evolution of hydrogen [16]. CPSA parameters were optimized as seen in Section 3.

2.4. Descriptive Statistics and Estimation of Detection Limit

Data were analyzed using MICROSOFT EXCEL (USA). Results are expressed as mean $\pm$ SD unless noted otherwise. The detection limits (3 signal/noise, S/N) were calculated

according to Long and Winefordner [28], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. Results and Discussion

The analysis of MMP raises methodical questions and concerns such as the type of sample (serum or plasma or blood), target molecule (total content of MMP or specific MPP) or form of MMP detected (MMP molecules or proMMP). Most commonly used methods for MMP analysis are immunochemistry and enzymatic-based ones [29].

3.1. Chronopotentiometric Stripping Analysis of MMP-9

Coupling adsorptive transfer stripping technique with chronopotentiometric stripping analysis has several advantages which include low detection limits for target molecules [9, 12, 13, 17, 23]. This coupled technique was employed to detect MMP-9 (Fig. 2A). Even though the amino acid cysteine forms only 3% (total count 19) of the total amino acid content in MMP-9 (total count 707), they are known to be responsible for most of the CPSA measured electroactivity of MMP-9. Experimental conditions were optimized to detect MMP-9 by AdTS CPSA. Time accumulation of MMP-9 onto HMDE was the first parameter optimized. Working MMP-9 concentrations used (1 ng/mL) were low, which showed high CPSA sensitivity to proteins. Measurements were carried out in borate buffer (pH 7.6) according to previously published results [12, 15, 16, 30]. MMP-9 was adsorbed onto HMDE at various times of accumulation: 30, 60, 90, 120, 150 and 180 s. Dependence of peak height on accumulation time is shown in Figure 2B. Peak height enhanced up to 90 s, and then decreased more than 50% possibly due to increase formation of complex structures on the working electrode's surface. Similar behavior of protein adsorption on HMDE surface was observed by Petrlova et al. [13, 31] and Adam et al. [32].

The pH of the supporting electrolyte plays an essential role in CPSA analysis. Hence, the electrochemical behavior of MMP-9 was studied in the following buffers: acetate buffer (pH 4.6), Britton – Robinson buffer (pH 6.5), phosphate buffer (pH 6.95) and borate buffer (7.6). MMP-9 signals measured in the buffers are shown in Figure 2C. MMP-9 gave signals at different potentials according to the pH and type of buffer: acetate buffer at -1.47 V; Britton – Robinson buffer at -1.62 V, phosphate buffer at -1.71 V and borate buffer at -1.74 V. The highest MMP-9 signal response was detected in acetate buffer (Fig. 2D). The lower pH values were determined to be more suitable for metalloproteinase electrochemical analysis than higher pH values. The value of MMP-9 isoelectric point is roughly 5.7 (http://www.signaling-gateway.org/molecule and is overall positively charged making MMP-9 detection advantageous. Fasciglione et al. showed experiments were unsuitable below pH 6.0 for accurate evaluation of MMP-9 enzymatic activity. Nevertheless, based on their results, MMP-9 is a relatively stable protein at pH values above 4.

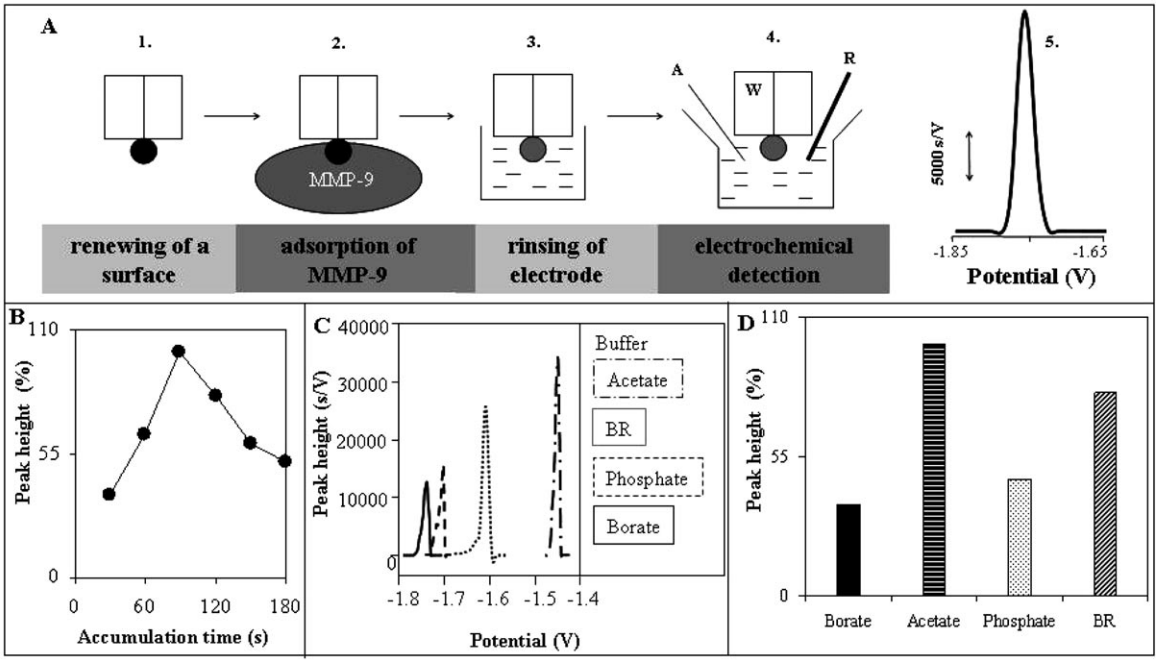

Fig. 2. A) Scheme of adsorptive transfer stripping technique used for the detection of collagen and/or MMP-9 or for the study of interactions between these molecules; 1) renewing of the hanging mercury drop electrode (HMDE) surface; 2) adsorption of MMP-9 or collagen in a drop solution onto the HMDE surface at open circuit; 3) rinsing electrode in water of ACS purity; 4) measuring by chronopotentiometric stripping analysis. B) Dependences of MMP-9 (1 ng/mL) peak height on accumulation time and C), D) type of supporting electrolyte.

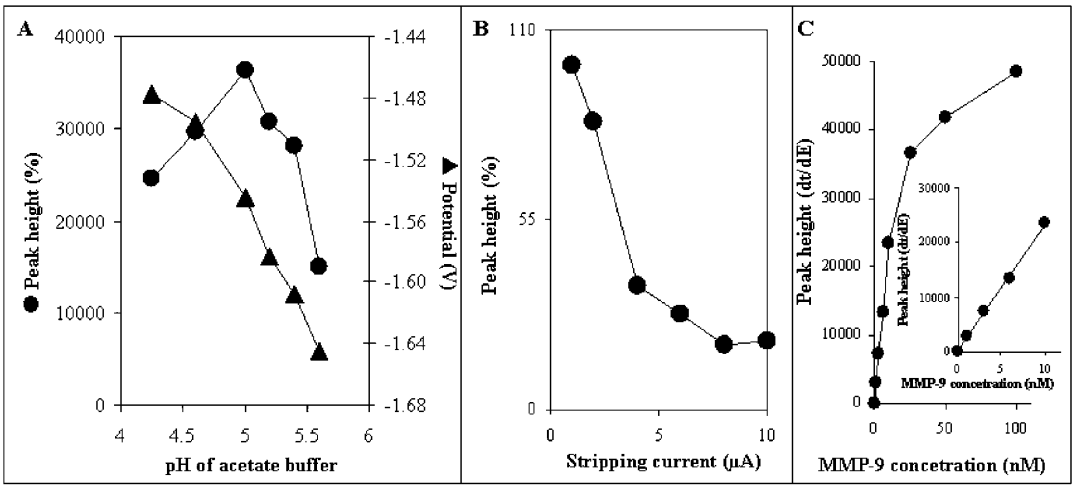

Fig. 3. Dependences of MMP-9 (1 ng/mL) peak height on A) pH of acetate buffer, B) stripping current and C) and inset, MMP-9 concentration.

The dependence of MMP-9 (1 ng/mL) peak height on various pH acetate buffers were investigated. This dependence is shown in Figure 3A. The highest CPSA response was observed at pH 5 0.2 M acetate buffer. Additionally, its potential shifted to more negative values with increasing pH. Lower pH values possibly facilitate hydrogen evolution from the supporting electrolyte during the catalytic reaction. Stripping current (1, 2, 4, 6, 8, 10 and 12 μA) was another experimental condition that influenced MMP-9 peaks (Fig. 3B). The lower stripping current resulted in a higher signal response. However, lower stripping currents (below 1 μA) produced lower reoccurring signals and increased relative standard deviations up to 10%. Based on results obtained a stripping current of 1 μA was selected for the following experiments.

The shape of the dependence of the CPSA peak height on MMP-9 concentration was obtained (Fig. 3C). Concentration ranging from 1 to 10 nM MMP-9 assigned a linear dependence ($y = 2299.6x + 220.36$, $R^2 = 0.9975$). The detection limit (3 *S*/*N*) was estimated to be 100 pM.

3.2. Collagen Modified HMDE

Preparation of standard collagen solutions is a difficult task. Dissolving collagen in water is limited due to its low solubility and its compact structure. With agitation and stirring the solubility of collagen can be enhanced, but the natural folding structure could be lost. Estimating collagen concentration by using spectrometry is also difficult. Electrochemical methods including chronopotentiometry are convenient alternative methods for estimating collagen concentration. The effect of two solvents, deionized water and HCl (9% *m*/*m*), tested solubility properties of collagen. Collagen suspensions were agitated using Vortex 2 (Eppendorf, Germany) at 400 rpm for 15 min. Collagen decomposition improved in HCl compared to water (Fig. 4A).

The effect of hydrochloric acid on collagen solubility was studied in greater detail. HCl solutions with concentrations ranging from 0.1 to 20% (*m*/*m*) were used to dissolve 100 mg of collagen. This solution (1 mL) was placed onto a shaker and agitated for 30 min. at 400 rpm. Collagen disintegration increased with increasing hydrochloric acid concentration. However acidic conditions (pH 0.5 – 1.5), can negatively influence the native structure of a protein. Usha and Ramasami found charge repulsion disrupts the stability of rat tail tendon collagen fiber at low pH values [33]. At pH lower then 6, there is a significant decrease in shrinkage temperature. This may partly be due to osmotic forces that lead to acid swelling. Extensive hydration could lead to significant volume changes and the rupture of the matrix structure. Furthermore, protonation of the ionizable group may dominate at pH values lower than the isoelectric point which could decrease intermolecular ion pair formation. Lower pH does not digest collagen fibers although MMP does. Collagen was dissolved with 9% HCl (*m*/*m*) and used in the following experiments.

3.3. Interaction of MMP-9 with Collagen

This work studied the interaction of MMP-9 with collagen using AdTS CPSA. The dependence of collagen peak height (1 μg/mL) on time accumulation is shown in Figure 4B. The highest response was measured at 90 s. In order to maintain the optimum conditions for the enzymatic collagen cleavage by MMP-9, MMP-9 dissolving solution, which contained 0.05 M Tris-HCl pH 7.6 + 0.2 M NaCl + 0.01 M $CaCl_2$, was used. At this pH, MMP-9 is activated and cleaves collagen [34]. MMP-9 concentration of 1 ng/mL gave a signal at − 1.65 V (Fig. 4C). The signal of collagen appeared at slightly more positive potentials (− 1.64 V). The interaction itself was studied according to the following scheme: i) HMDE surface was renewed. ii) Collagen (1 μg/mL) accumulated (90 s) on renewed HMDE surface at open

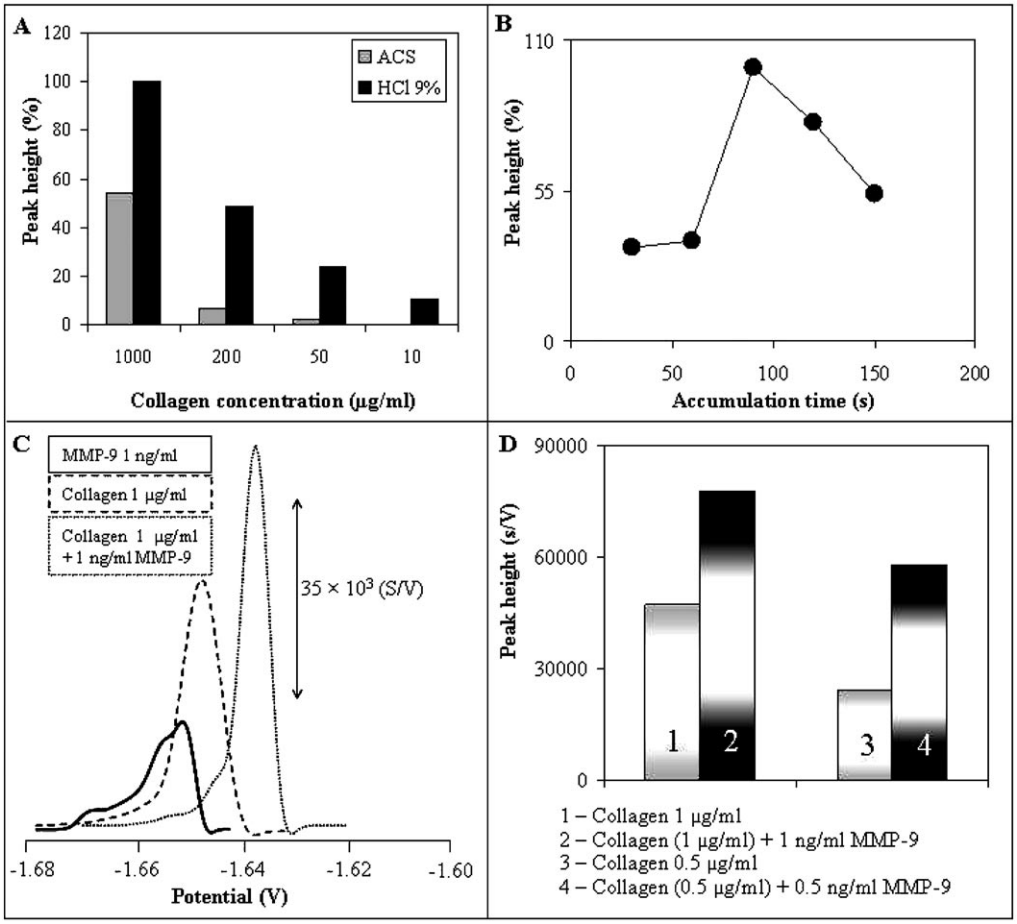

Fig. 4. A) Height of peaks of collagen dissolved in ACS water or 9% HCl. B) Dependence of collagen peak height on accumulation time. C) Signals of collagen, MMP-9 and collagen after interaction with MMP-9 measured by AdTS CPSA (interaction time: 30 s). D) Height of CPSA peaks of collagen (0.5 or 1 µg/mL) after interaction with MMP-9 (0.5 or 1 ng/mL).

circuit. iii) The electrode was rinsed in ACS grade water and immersed in 5 µL MMP-9 solution (1 ng/mL). iv) The interaction between MMP-9 and collagen was studied from 30 to 300 s under open circuit. v) The electrode was then rinsed in ACS water. vi) The electrode was transferred into an electrochemical cell and measured in acetate buffer (pH 5). The change in CPSA peak is shown in Figure 4C. CPSA signal of collagen after interaction with MMP-9 increased more than 30% compared to CPSA signal of collagen only. The potential of the signal was shifted 20 mV toward positive values. With increased MMP-9 interaction, the signal of collagen adsorption onto HMDE enhanced. The experiment was repeated with lower collagen and MMP-9 concentrations. The concentration of both components were halved: 0.5 µg/mL collagen itself (Fig. 4D-column 3) and collagen after interaction with MMP-9 (0.5 ng/mL) (Fig. 4D-column 4). The signals measured were cut in half compared to previous results. Based on the obtained results, it is possible collagen is cleaved into smaller fragments by MMP-9. These fragments are conveniently accessible to the HMDE's surface, resulting in a higher signal (Fig. 4D). Similar phenomenon were observed during the analysis of denatured protein p53 [35, 36], urease [37] and lactoferrin [38 – 40].

4. Conclusions

Study of protein-protein interactions in the past have required expensive, time consuming and labor intensive methods, techniques and approaches. Adsorptive transfer stripping technique coupled with chronopotentiometric stripping analysis is an easy and low cost approach to detect MMP-9 interaction with collagen. This technique determines the cleavage of collagen catalyzed by MMP-9 using enhanced CPSA signals. The well observed signal is probably due to the collagen moieties open access to the electrode's surface.

5. Acknowledgements

Financial support from the Grants IGA MZLU MP 12/AF and 2A-1591/122-MPO is highly acknowledged. The au-

thors wish to express their thanks to Dr. Grace Chavis for English correction and discussion.

6. References

[1] H. Nagase, J. F. Woessner, *J. Biol. Chem.* **1999**, *274*, 21491.
[2] H. J. Ra, W. C. Parks, *Matrix Biol.* **2007**, *26*, 587.
[3] P. L. Jones, F. S. Jones, *Matrix Biol.* **2000**, *19*, 581.
[4] S. Ye, *Matrix Biol.* **2000**, *19*, 623.
[5] G. Murphy, V. Knauper, *Matrix Biol.* **1997**, *15*, 511.
[6] P. Basset, A. Okada, M. P. Chenard, R. Kannan, I. Stoll, P. Anglard, J. P. Bellocq, M. C. Rio, *Matrix Biol.* **1997**, *15*, 535.
[7] H. E. Vanwart, H. Birkedalhansen, *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 5578.
[8] J. W. Becker, A. I. Marcy, L. L. Rokosz, M. G. Axel, J. J. Burbaum, P. M. D. Fitzgerald, P. M. Cameron, C. K. Esser, W. K. Hagmann, J. D. Hermes, J. P. Springer, *Protein Sci.* **1995**, *4*, 1966.
[9] E. Palecek, V. Ostatna, *Electroanalysis* **2007**, *19*, 2383.
[10] M. Tomschik, L. Havran, M. Fojta, E. Palecek, *Electroanalysis* **1998**, *10*, 403.
[11] R. Selesovska-Fadrna, M. Fojta, T. Navratil, J. Chylkova, *Anal. Chim. Acta* **2007**, *582*, 344.
[12] R. Kizek, L. Trnkova, E. Palecek, *Anal. Chem.* **2001**, *73*, 4801.
[13] J. Petrlova, S. Krizkova, O. Zitka, J. Hubalek, R. Prusa, V. Adam, J. Wang, M. Beklova, B. Sures, R. Kizek, *Sens. Actuators B, Chem.* **2007**, *127*, 112.
[14] I. Sestakova, M. Kopanica, L. Havran, E. Palecek, *Electroanalysis* **2000**, *12*, 100.
[15] M. Strouhal, R. Kizek, J. Vecek, L. Trnkova, M. Nemec, *Bioelectrochemistry* **2003**, *60*, 29.
[16] L. Trnkova, R. Kizek, J. Vacek, *Bioelectrochemistry* **2002**, *56*, 57.
[17] S. Krizkova, O. Zitka, V. Adam, M. Beklova, A. Horna, Z. Svobodova, B. Sures, L. Trnkova, L. Zeman, R. Kizek, *Czech J. Anim. Sci.* **2007**, *52*, 143.
[18] I. Fabrik, S. Krizkova, D. Huska, V. Adam, J. Hubalek, L. Trnkova, T. Eckschlager, J. Kukacka, R. Prusa, R. Kizek, *Electroanalysis* **2008**, *20*, 1521.
[19] M. Masarik, A. Stobiecka, R. Kizek, F. Jelen, Z. Pechan, W. Hoyer, T. M. Jovin, V. Subramaniam, E. Palecek, *Electroanalysis* **2004**, *16*, 1172.
[20] E. Palecek, M. Masarik, R. Kizek, D. Kuhlmeier, J. Hassmann, J. Schulein, *Anal. Chem.* **2004**, *76*, 5930.
[21] M. Brazdova, R. Kizek, L. Havran, E. Palecek, *Bioelectrochemistry* **2002**, *55*, 115.
[22] J. L. A. Sanchez, E. Baldrich, A. E. G. Radi, S. Dondapati, P. L. Sanchez, I. Katakis, C. K. O'Sullivan, *Electroanalysis* **2006**, *18*, 1957.
[23] V. Ostatna, E. Palecek, *Electrochim. Acta* **2008**, *53*, 4014.
[24] V. Ostatna, B. Uslu, B. Dogan, S. Ozkan, E. Palecek, *J. Electroanal. Chem.* **2006**, *593*, 172.
[25] N. Serrano, I. Sestakova, J. M. Diaz-Cruz, *Electroanalysis* **2006**, *18*, 169.
[26] N. Serrano, I. Sestakova, J. M. Diaz-Cruz, C. Arino, *J. Electroanal. Chem.* **2006**, *591*, 105.
[27] V. Dorcak, E. Palecek, *Electroanalysis* **2007**, *19*, 2405.
[28] G. L. Long, J. D. Winefordner, *Anal. Chem.* **1983**, *55*, A712.
[29] C. Lombard, J. Saulnier, J. Wallach, *Biochimie* **2005**, *87*, 265.
[30] R. Prusa, R. Kizek, L. Trnkova, J. Vacek, J. Zehnalek, *Clin. Chem.* **2004**, *50*, A28.
[31] J. Petrlova, D. Potesil, R. Mikelova, O. Blastik, V. Adam, L. Trnkova, F. Jelen, R. Prusa, J. Kukacka, R. Kizek, *Electrochim. Acta* **2006**, *51*, 5112.
[32] V. Adam, S. Krizkova, O. Zitka, L. Trnkova, J. Petrlova, M. Beklova, R. Kizek, *Electroanalysis* **2007**, *19*, 339.
[33] R. Usha, T. Ramasami, *J. Appl. Polym. Sci.* **2000**, *75*, 1577.
[34] G. F. Fasciglione, S. Marini, S. D'Alessio, V. Politi, M. Coletta, *Biophys. J.* **2000**, *79*, 2138.
[35] D. Potesil, R. Mikelova, V. Adam, R. Kizek, R. Prusa, *Protein J.* **2006**, *25*, 23.
[36] R. Prusa, D. Potesil, M. Masarik, V. Adam, R. Kizek, F. Jelen, *Mol. Biol. Cell*. **2004**, *15*, 249A.
[37] J. Hubalek, J. Hradecky, V. Adam, O. Krystofova, D. Huska, M. Masarik, L. Trnkova, A. Horna, K. Klosova, M. Adamek, J. Zehnalek, R. Kizek, *Sensors* **2007**, *7*, 1238.
[38] V. Adam, O. Zitka, P. Dolezal, L. Zeman, A. Horna, J. Hubalek, J. Sileny, S. Krizkova, L. Trnkova, R. Kizek, *Sensors* **2008**, *8*, 464.
[39] O. Zitka, A. Horna, K. Stejskal, J. Zehnalek, V. Adam, L. Havel, L. Zeman, R. Kizek, *Acta Chim. Slov.* **2007**, *54*, 68.
[40] J. Kukacka, O. Zitka, A. Horna, K. Stejskal, J. Zehnalek, V. Adam, L. Havel, L. Zeman, R. Prusa, L. Trnkova, R. Kizek, *Faseb J.* **2007**, *21*, A635.

