Scientific paper

Study of Structural Changes of Lactoferrin Using Flow Injection Analysis with Electrochemical Detection on Glassy Carbon Electrode

Ondrej Zitka,[a,b] Ales Horna,[c] Karel Stejskal,[a,b] Josef Zehnalek,[a] Vojtech Adam,[a] Ladislav Havel,[d] Ladislav Zeman,[e] Rene Kizek[a,*]

[a] *Department of Chemistry and Biochemistry,* [d] *Department of Plant Biology, and* [e] *Department of Animal Nutrition, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic. Tel. +420(5)45133350, Fax: +420(5)45212044, E-mail: kizek@sci.muni.cz.*

[b] *Department of Biochemistry, Faculty of Science, Masaryk University, Kotlarska 2, CZ-611 37 Brno, Czech Republic*

[c] *Department of Food Engineering and Chemistry, Faculty of Technology, Tomas Bata University, CZ-762 72 Zlin, Czech Republic*

Received: 09-10-2006

Paper based on a presentation at the 12th International Symposium on Separation Sciences, Lipica, Slovenia, September 27–29, 2006.

Abstract

Lactoferrin is considered to be a multifunctional protein. It appears to play several biologically important roles, where its structure is very crucial. The aim of this work was to investigate the basic electrochemical behaviour of lactoferrin by both stationary and flow electrochemical methods with respect its structural changes under various denaturing conditions. The electroactivity of lactoferrin was studied by three various electrochemical methods (linear sweep, differential pulse and square wave voltammetry). Based on the results obtained, we utilized flow injection analysis with electrochemical detection (FIA-ED) for determination of lactoferrin. We found out that the most suitable FIA-ED conditions were as follows: working electrode potential of 900 mV, Britton-Robinson buffer (pH 4.5) as the mobile phase, its flow rate 1 ml min^{-1}. Finally, we attempted to follow the changes of lactoferrin signal in the presence of chemical compounds or under the physical conditions leading to changes in its structure. As we have shown here, electrochemical analysis enables us to distinguish changes of protein structure easily and rapidly.

Keywords: lactoferrin, milk protein, linear sweep voltammetry, differential pulse voltammetry, square wave voltammetry, carbon paste electrode, glassy carbon electrode

1. Introduction

Milk represents one of staple foods in human nutrition. Its biological and chemical composition have been investigating intensively.[1–4] Based on the results obtained from these investigations, milk is by far the most abundant source of the protein called lactoferrin, e.g. colostrum contains up to 7 g of this compound per litre.[5] Lactoferrin itself was discovered in 1939 in cow milk. Its name is derived from its former classification as the crucial iron-binding protein in milk.[6–8] In 1960, lactoferrin was first isolated from human breast milk.[9] Since then this protein has been found on mucosal surfaces, within the specific granules of polymorphonuclear leukocytes, in saliva, tears and seminal fluids, indicating that it could play a protective role in the innate immune response.[10–12]

Lactoferrin is a glycoprotein consisted from 703 aminoacid residues. Hololactoferrin is formed from one linear polypeptide chain forming two spherical domains (*C*- and *N*-terminal), each domain contains one iron binding site. Its molecular weight is of about 77,000 Da. Lactoferrin is able to bind other metals and their oxides (e.g. Ga^{3+}, Al^{3+}, VO^{2+}, Mn^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+}, and trivalent lanthanoids) into the same binding place, but with lower affinity. The molecular weight and structure of lactoferrin is similar to other iron-binding proteins, transferrins.

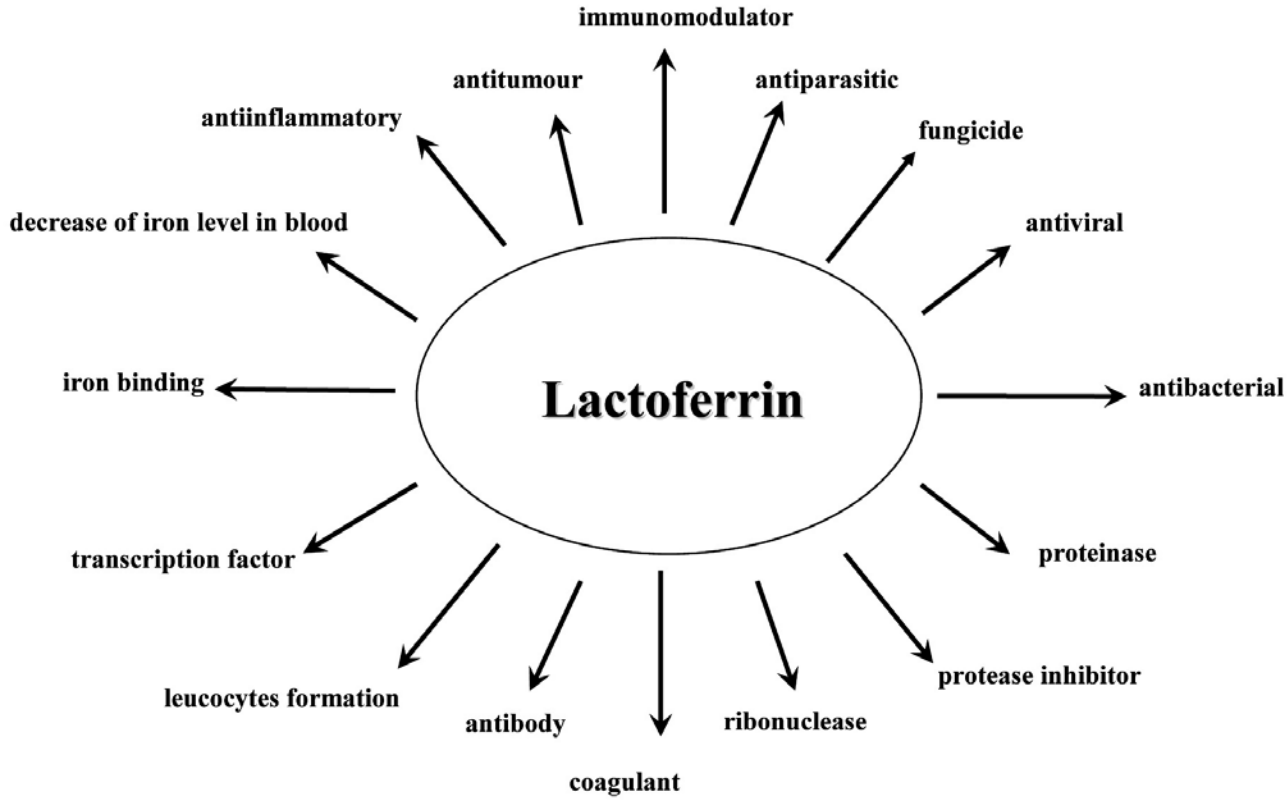

Figure 1. Scheme of the many biologically important functions of lactoferrin.

Thanks to this fact lactoferrin belongs to the transferrin family.[13] The plasmatic lactoferrin is synthesized by leucocytes.

The main biological function of lactoferrin is to maintain iron homeostasis. The binding of iron by lactoferrin proceeds primarily in the intestine. Moreover it can play important roles in cell growth regulation, cell differentiation and itself has various functions.[8,14–19] For review of lactoferrin function see the scheme (Figure 1). Based on the functions of this protein, lactofferin could be proposed as an antioxidant[20,21] and could be associated with tumour diseases progression and metastases.[22]

Electrochemical techniques are an attractive alternative method for detection of electroactive species, because of its inherent advantages of simplicity, ease of miniaturization, high sensitivity and relatively low cost.[23–33] The aim of this work was to investigate basic electrochemical behaviour of lactoferrin by both stationary and flow electrochemical methods with respect to study its structural changes under various denaturing conditions.

2. Materials and Methods

2.1. Chemicals

Acetonitrile and methanol (HPLC-purity) were obtained from Merck (Darmstadt, Germany). The DMW lactoferrin standard was purchased from NUTRA ingrediens (Netherlands). All other chemicals used were from Sigma-Aldrich (USA) unless noted otherwise. The standard stock solutions of lactoferrin (concentration of 1 mg mL^{-1}) were prepared in ACS water (Aldrich, USA) and stored in dark at 4 °C.

2.2. Stationary Electrochemical Measurement

The electrochemical measurement were performed using an AUTOLAB analyzer (EcoChemie, Netherlands) in connection with VA-Stand 663 (Metrohm, Switzerland) using a standard cell with three electrodes. The electrode system consisted of a carbon paste working electrode, an Ag/AgCl/3 M KCl reference electrode, and a graphite counter electrode. The carbon paste (about 0.5 g) was made of 70% graphite powder (Sigma-Aldrich) and 30% mineral oil (Sigma-Aldrich; free of DNase, RNase, and protease). This paste was housed in a Teflon body having a 2.5-mm-diameter disk surface. Prior to measurements, an electrode surface was renewed by polishing with a soft filter paper. Then, the surface was ready for measurement of a sample volume of 5 μL.[34–36] All electrochemical measurements were carried out in the presence of acetate buffer pH = 5.0 at room temperature. The analyzed samples were deoxygenated with argon (99.999%) saturated with water for 120 s. The voltammetric parameters were as fol-

lows: Linear sweep voltammetry: initial potential of 0 V, end potential 1.5 V, step potential 5 mV. Differential pulse voltammetry: initial potential of 0 V, end potential 1.5 V, step potential 5 mV. Square wave voltammetry: initial potential of 0 V, end potential 1.5 V, frequency 240 Hz.

2.3. Flow Injection Analysis with Electrochemical Detection

A flow injection analysis with electrochemical detection (FIA-ED) consisted of solvent delivery pump operating in range of 0.001–9.999 mL min^{-1} (Model 583 ESA Inc., Chelmsford, MA, USA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m) and an electrochemical detector. The electrochemical detector includes one low volume flow-through analytical cells (Model 5040, ESA, USA), which is consisted of glassy carbon working electrode, hydrogen-palladium electrode as reference electrode and auxiliary carbon electrode, and Coulochem III as a control module. The sample (5 μL) was injected manually. The obtained data were treated by CSW 32 software. The experiments were carried out at room temperature. Guard cell potential was 0 V. A glassy carbon electrode was polished mechanically by 0.1 μm of alumina (ESA Inc., USA) and sonicated at the room temperature for 5 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W.

2.4. Denaturation of Lactoferrin

Heat denaturation: lactoferrin (1 mg mL^{-1}) in the presence of Britton-Robinson was denatured using thermomixer Eppendorf at 65 °C for 30 min with occasional vortexing (400 rpm). Methanol denaturation: lactoferrin (about 1 mg) was weighed (Sartorius), transferred to a micro-tube with 1 mL of methanol (99.8%, Sigma Aldrich, HPLC grade) and placed on Vortex (Genie, USA) with occasionally vortexing (400 rpm) for 30 min. Urea denaturation: lactoferrin (1 mg mL^{-1}) was mixed with urea (6 mol L^{-1}) and placed on a thermomixer Eppendorf at 65 °C for 30 min with occasionally vortexing (400 rpm). At the end of the denaturing, the samples were collected prior to analysis by FIA-ED.

3. Results and Discussion

3.1. Electrochemical Behaviour of Lactoferrin on Carbon Paste Electrode Surface

Due to the fundamental character of this study, stationary electrochemical methods using a standard cell with three electrodes (carbon paste working electrode – CPE, an Ag/AgCl/3 mol L^{-1} KCl reference electrode, and a graphite counter electrode) were utilized. We studied electroactivity of lactoferrin (1 μg mL^{-1}) in the presence of acetate buffer (pH 5.0) using three various electrochemical methods (linear sweep – LSV, differential pulse – DPV and square wave voltammetry – SWV). The oxidation signals were observed at the potentials of 0.8–0.85 V according to electrochemical methods used (Figure 2). The signals were well developed and distinguishable.

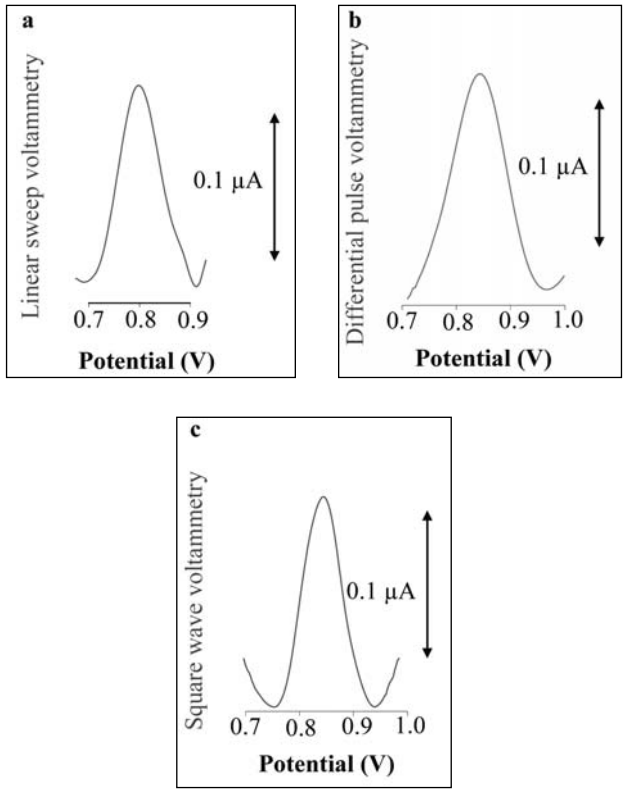

Figure 2. Signal of lactoferrin measured by (a) linear sweep; (b) differential pulse; (c) square wave voltammetry. Lactoferrin concentration: 100 μg mL^{-1}, supporting electrolyte: acetate buffer (pH 4.0), working electrode: CPE. Measurements were carried out using AUTOLAB analyser. Parameters of the methods as follows: LSV – initial potential of 0 V, end potential 1.5 V, step potential 5 mV, scan rate 50 mV s^{-1}; DPV – initial potential of 0 V, end potential 1.5 V, step potential 5 mV; SWV – initial potential of 0 V, end potential 1.5 V, frequency 240 Hz. Experimental data were baseline-corrected.

3.2. Flow Injection Analysis of Lactoferrin with Electrochemical Detection

Based on the results obtained above, where the electroactivity of lactoferrin has been demonstrated, FIA-ED was used for determination of lactoferrin. Glassy carbon electrode was used as working electrode. Primarily, we tested different buffers (acetate, phosphate and Britton-Robinson) for determination of lactoferrin at 700 mV and flow rate of 1 mL min^{-1}. It follows from the results obtained that lactoferrin gave a higher response (about 20–30%) in the presence of acetate buffer in comparison with phosphate buffer. Moreover, the highest response of

lactoferrin was measured in the presence of Britton-Robinson buffer. Thus, we utilized Britton-Robinson buffer as the mobile phase for determination of lactoferrin by FIA-ED for characterization of the compound of interest. Due to the choice of Britton-Robinson buffer as mobile phase, we were interested in the issue how could pH of this buffer influence the lactoferrin response. Thus, the study of influence of pH of this buffer within the range from 3.5 to 6.5 on response of lactoferrin followed (Figure 2A). The response increased till pH of 4.5 and then decreased. In conclusion, we chose Briton-Robinson buffer (pH 4.5) as the mobile phase for detection of lactoferrin.

Besides optimisation of flow conditions, we attempted to optimise the detection potential. Based on the results obtained by voltammetric techniques, we expected the highest response at the potential of about 800 mV. We observed changes in lactoferrin response within the range from 400 to 900 mV. The electrochemical signal increased rapidly with increasing potential applied up to 500–600 mV and then increased more gradually. For the following measurements we utilized potential of 900 mV due to the highest response and low standard deviation (about 3–5% (Figure 2B). The difference between the potential of the peak maxima measured by stationary electrochemical system and of the maximum measured by flow electrochemical technique can be associated with (i) different working electrodes (carbon paste vs. glassy carbon) and (ii) different reference electrodes (Ag/AgCl 3M KCl vs. hydrogen-palladium electrode).

Using the optimized experimental conditions, the dependence of lactoferrin response on its concentrations was studied. The FIA-ED records of lactoferrin (8, 16, 32 and 64 μg mL^{-1}) are shown in Figure 3C. The calibration dependence plotted were linear within range from 2.5 to 60 μg mL^{-1} (y = 0.4689x + 1.0096; R^2 = 0.9939).

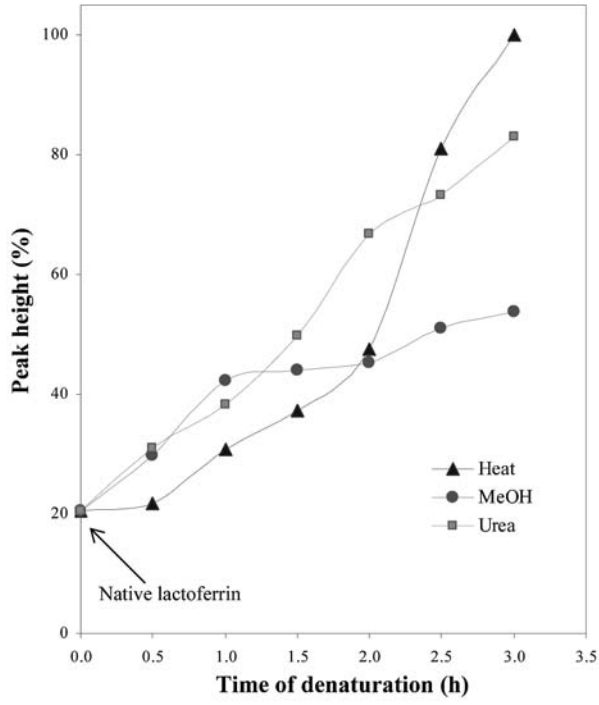

Figure 4. Effects of heat, urea or methanol on lactoferrin signal. Lactoferrin concentration: 1 mg mL^{-1}. For other experimental conditions see Figure 3 and Materials and methods.

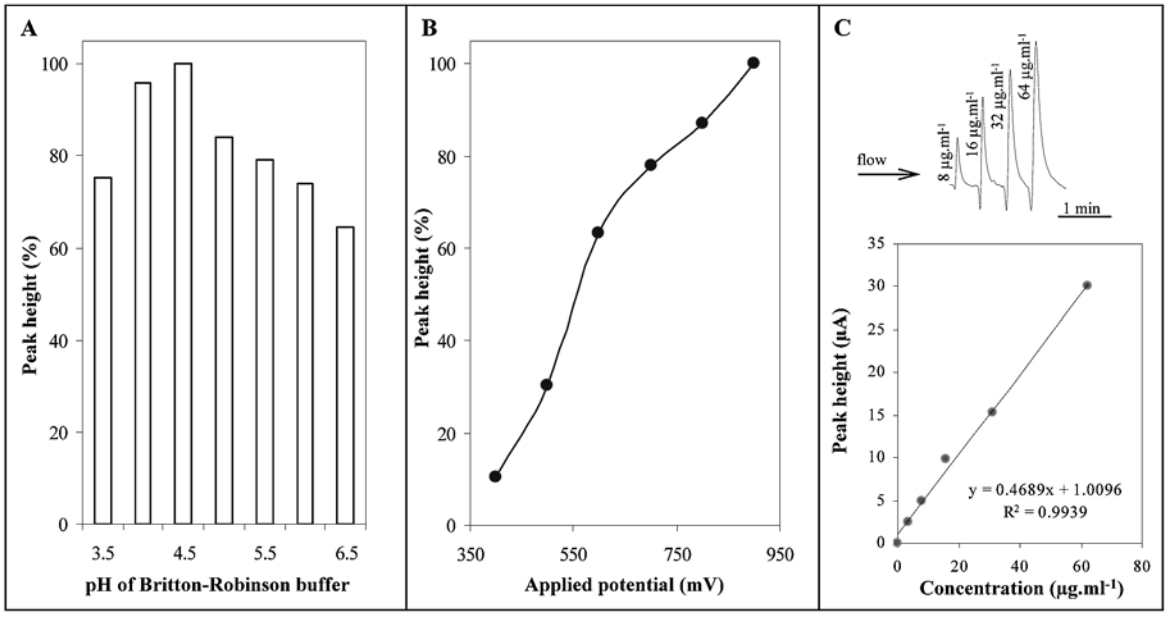

Figure 3. Influence of pH of Britton-Robinson buffer on lactoferrin signal; working electrode potential: 700 mV (A). Hydrodynamic voltammogram (B). Dependence of lactoferrin signal on its concentration (C); inset: FIA-ED records of lactoferrin (8, 16, 32 and 64 μg mL^{-1}). Lactoferrin concentration: 100 μg mL^{-1}, mobile phase: Britton-Robinson buffer, working electrode: GC; measurement were carried out using CoulochemIII. The FIA-ED conditions were as follows: applied potential 900 mV, current range 1 μA, flow of mobile phase 1 mL min^{-1}; temperature 22 °C.

3.3. Distinguishing the Native and Denatured Lactoferrin

To follow the denaturation of proteins, e.g. large proteins with molecular weight of several 10 kDa, is a difficult task. There are no simple analytical and biochemical tools for this purpose. Here, we attempted to follow lactoferrin signal in the presence of chemical compounds or under the physical conditions leading to changes in its structure (Figure 3). We measured the signal of denaturing lactoferrin three times per hour for three hours. The analyses of native lactoferrin was performed with a standard deviation of 5–6%). During heat denaturation, lactoferrin was denatured under 65 °C, whereas a gradual increase of its signal was observed for up to 2 h of heating and then the signal increased rapidly. This phenomenon can be associated with a structural change occurring after 2-h heating. Besides, if we utilized the commonly used denaturing compounds such as 6 mol L^{-1} urea at 37 °C, the signal of lactoferrin was proportional to the time of this chemical denaturation. This lactoferrin behaviour shows that its structure can be released very gradually under this condition. Moreover, under the effect of methanol, the signal of lactoferrin increased up to 1 h and then the signal changed gradually (Figure 3). On the other hand, this protein precipitated in the presence of methanol, thus the real concentration of free lactoferrin was doubtful.

4. Conclusion

To distinguish the native and the denatured structural form of a protein is still a very difficult task. As we have shown here, electrochemical analysis enables us to distinguish a change of protein structure easily and rapidly. The changes observed using electroanalytical techniques can be associated with the release of aminoacid moieties, which can interact with the surface of a working electrode. Moreover, the coupling of an electrochemical method and flow injection analysis leads to new possibilities for routine analysis.

5. Acknowledgement

The work on this project was supported by grants FI-IM/076, MSMT 1M06030 and INCHEMBIOL MSMT 0021622412.

6. References

1. P. Hodbod, L. Zeman, *Czech J. Anim. Sci.* **2001**, *46*, 509–513.
2. S. Kracmar, S. Gajdusek, P. Jelinek, L. Zeman, V. Kozel, M. Kozlova, E. Kracmarova, *Czech J. Anim. Sci.* **1999**, *44*, 541–545.
3. S. Kracmar, S. Gajdusek, J. Kuchtik, L. Zeman, F. Horak, G. Doupovcova, R. Matejkova, E. Kracmarova, *Czech J. Anim. Sci.* **1998**, *43*, 369–374.
4. S. Kracmar, S. Gajdusek, J. Kuchtik, L. Zeman, F. Horak, G. Doupovcova, E. Kracmarova, *Czech J. Anim. Sci.* **1998**, *43*, 251–255.
5. S. Farnaud, R. W. Evans, *Mol. Immunol.* **2003**, *40*, 395–405.
6. L. Sanchez, P. Aranda, M. D. Perez, M. Calvo, *Biological Chemistry Hoppe-Seyler* **1988**, *369*, 1005–1008.
7. L. Sanchez, M. Calvo, J. H. Brock, *Arch. Dis. Child.* **1992**, *67*, 657–661.
8. L. Sanchez, J. M. Peiro, H. Castillo, M. D. Perez, J. M. Ena, M. Calvo, *J. Food Sci.* **1992**, *57*, 873–879.
9. A. Bezkorovainy, *J. Dairy Sci.* **1977**, *60*, 1977.
10. V. Bernal, P. Jelen, *J. Dairy Sci.* **1985**, *68*, 2847–2852.
11. E. N. Baker, H. M. Baker, *CMLS-Cell. Mol. Life Sci.* **2005**, *62*, 2531–2539.
12. H. M. Baker, E. N. Baker, *Biometals* **2004**, *17*, 209–216.
13. P. F. Levay, M. Viljoen, *Haematologica* **1995**, *80*, 252–267.
14. D. Legrand, E. Elass, M. Carpentier, J. Mazurier, *CMLS-Cell. Mol. Life Sci.* **2005**, *62*, 2549–2559.
15. Y. Pan, J. Wan, H. Roginski, A. Lee, B. Shiell, W. P. Michalski, J. Coventry, *Aust. J. Dairy Technol.* **2005**, *60*, 149–151.
16. J. M. Steijns, A. C. M. van Hooijdonk, *Br. J. Nutr.* **2000**, *84*, S11–S17.
17. P. P. Ward, C. S. Piddington, G. A. Cunningham, X. D. Zhou, R. D. Wyatt, O. M. Conneely, *Bio-Technology* **1995**, *13*, 498–503.
18. J. G. Ruiz-Pelaez, S. Mattar, *Pediatr. Infect. Dis. J.* **1999**, *18*, 342–346.
19. O. Saitoh, K. Kojima, M. Kayazawa, K. Sugi, S. Tanaka, K. Nakagawa, T. Teranishi, R. Matsuse, K. Uchida, H. Morikawa, I. Hirata, K. Katsu, *Intern. Med.* **2000**, *39*, 778–782.
20. J. Chen, L. Gorton, B. Akesson, *Anal. Chim. Acta* **2002**, *474*, 137–146.
21. J. Chen, H. Lindmark-Mansson, L. Gorton, B. Akesson, *Int. Dairy J.* **2003**, *13*, 927–935.
22. P. P. Ward, E. Paz, O. M. Conneely, *CMLS-Cell. Mol. Life Sci.* **2005**, *62*, 2540–2548.
23. E. Palecek, M. Masarik, R. Kizek, D. Kuhlmeier, J. Hassmann, J. Schulein, *Anal. Chem.* **2004**, *76*, 5930–5936.
24. M. Masarik, A. Stobiecka, R. Kizek, F. Jelen, Z. Pechan, W. Hoyer, T. M. Jovin, V. Subramaniam, E. Palecek, *Electroanalysis* **2004**, *16*, 1172–1181.
25. R. Kizek, J. Vacek, L. Trnkova, B. Klejdus, L. Havel, *Chem. Listy* **2004**, *98*, 166–173.
26. M. Masarik, R. Kizek, K. J. Kramer, S. Billova, M. Brazdova, J. Vacek, M. Bailey, F. Jelen, J. A. Howard, *Anal. Chem.* **2003**, *75*, 2663–2669.
27. S. Billova, R. Kizek, E. Palecek, *Bioelectrochemistry* **2002**, *56*, 63–66.
28. R. Kizek, L. Trnkova, S. Sevcikova, J. Smarda, F. Jelen, *Anal. Biochem.* **2002**, *301*, 8–13.

29. R. Kizek, L. Trnkova, E. Palecek, *Anal. Chem.* **2001**, *73*, 4801–4807.
30. L. Trnkova, F. Jelen, J. Petrlova, V. Adam, D. Potesil, R. Kizek, *Sensors* **2005**, *5*, 448–464.
31. J. Vacek, Z. Andrysik, L. Trnkova, R. Kizek, *Electroanalysis* **2004**, *16*, 224–230.
32. S. Billova, R. Kizek, F. Jelen, P. Novotna, *Anal. Bioanal. Chem.* **2003**, *377*, 362–369.
33. J. Petrlova, R. Mikelova, K. Stejskal, A. Kleckerova, O. Zitka, J. Petrek, L. Havel, J. Zehnalek, V. Adam, L. Trnkova, R. Kizek, *J. Sep. Sci.* **2006**, *29*, 1166–1173.
34. D. Potesil, R. Mikelova, V. Adam, R. Kizek, R. Prusa, *Protein J.* **2006**, *25*, 23–32.
35. R. Kizek, M. Masarik, K. J. Kramer, D. Potesil, M. Bailey, J. A. Howard, B. Klejdus, R. Mikelova, V. Adam, L. Trnkova, F. Jelen, *Anal. Bioanal. Chem.* **2005**, *381*, 1167–1178.
36. R. Prusa, D. Potesil, M. Masarik, V. Adam, R. Kizek, F. Jelen, *Mol. Biol. Cell* **2004**, *15*, 249A–249A.

Povzetek

Laktoferin je večfunkcionalna beljakovina in igra več biološko pomembnih vlog, pri katerih je struktura beljakovine zelo pomembna. Raziskali smo nekatere elektrokemijske lastnosti laktoferina tako z uporabo stacionarnih kot tudi pretočnih tehnik, zlasti z ozirom na strukturne spremembe med denaturacijo. Elektroaktivnost smo spremljali s tremi voltametričnimi tehnikami. Na podlagi teh rezultatov smo uvedli pretočno analizno metodo z elektrokemijsko detekcijo za določanje laktoferina. Ugotovili smo naslednje optimalne analizne parametre: potencial delovne elektrode 900 mV, Britton-Robinsonov pufer kot mobilna faza (pH 4,5) in pretok 1 mL min^{-1}. Med denaturacijo smo sledili tudi spremembam signala, ki je odgovarjal laktoferinu. Tem spremembam lahko s predlagano elektrokemijsko metodo sledimo zlahka in hitro.