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Research Article

Separation of tryptic peptides of native and glycated BSA using open-tubular CEC with salophene–lanthanide–Zn^{2+} complex as stationary phase

Open-tubular CEC (OT-CEC) with a new stationary phase, salophene–lanthanide–Zn^{2+} complex, has been applied to the separation of tryptic peptides of native BSA and BSA glycated by glucose and ribose. Glycation of proteins (non-enzymatic modification by sugars) significantly affects their properties and it is of great importance from a physiological point of view. Separation of tryptic peptides of glycated BSA by CZE was poor because of their strong adsorption to the bare fused silica capillary. An improved separation of tryptic peptides of both native and glycated BSA was achieved by OT-CEC in the fused silica capillary non-covalently coated with salophene–lanthanide–Zn^{2+} complex, which suppressed the adsorption of peptides to the capillary and *via* specific interactions with some (glyco)peptides enhanced selectivity of the separation. Significant differences have been found in OT-CEC analyses of tryptic hydrolysates of native and glycated BSA. In OT-CEC-UV profile of tryptic peptides of native BSA, 44 peaks could be resolved, whereas a reduced number of 38 peaks were observed in the profile of tryptic peptides of glucose-glycated BSA and only 30 peaks were found in the case of ribose-glycated BSA. The developed OT-CEC can be potentially used for monitoring of protein glycation.

Keywords: BSA / Glycation / Open-tubular CEC / Peptide mapping / Salophene–lanthanide–Zn^{2+}complex
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1 Introduction

CEC is a hybrid separation technique, utilizing the principles of electromigration techniques (electroosmosis and electrophoresis) and chromatography (distribution between two phases). There are numerous books and review articles on the principles and applications of CEC [1–6]. CEC is typically performed in a fused silica (FS) capillary internally filled or coated with a chromatographic stationary phase. The EOF is used to drive the mobile phase and the differential interactions of the analytes with the stationary phase result in their separation. In general, a high separation efficiency of CEC separations is achieved, thanks to the almost piston-like profile of EOF. CEC thus initially gained recognition as an EOF-based method for efficient separation of neutral as well as charged compounds [7, 8]. In principle, CEC can be classified in two ways: according to column format and according to separation mechanism. Three CEC modes are distinguished based on the column format: columns packed with particles, monolithic columns and open-tubular systems (open-tubular CEC, OT-CEC). The second way of classification results from the separation mode: in principle, the separation modes in CEC are identical with those in classical LC, *i.e.* reversed phase, normal phase, ion-exchange, size-exclusion, or affinity modes are discerned, respectively.

OT-CEC represents a CEC mode based on the covalent bonding or non-covalent (physical) attachment of a thin layer stationary phase to the inner capillary wall. The first OT-CEC separation was reported by Tsuda *et al.* [9] using an octadecyl-modified glass capillary. Novel types of capillary columns for OT-CEC are based on etched chemically modified FS capillaries [10, 11] and on porous layer of monolithic polymer [12, 13].

In the current era of proteomics, the significant topics are applications of CEC and OT-CEC to the separation of complex protein and peptide mixtures, such as cell lysates, tissue extracts and body fluids, in the investigation of partial or total proteomes of particular organisms, organs, or cells; for the applications of CEC, OT-CEC and related electromigration techniques to separation and analysis of peptides and proteins, see the recent reviews [14–19].

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Abbreviations: FS, fused silica; **OT-CEC,** open-tubular CEC

CEC and OT-CEC allow very specific and efficient separations of closely related analytes. This is an important aspect also in the area of peptide mapping of large proteins, *i.e.* separation of complex peptide mixtures originating from the chemical and enzymatic hydrolysis of proteins. In this study, we have tried to improve the separability of such complex protein hydrolysates by employing OT-CEC with a new stationary phase, salophene–lanthanide–Zn^{2+} complex (Fig. 1), non-covalently attached *via* physical adsorption to the inner FS capillary wall. Tryptic peptides of BSA were used as model peptide mixture.

Salophens represent a special class of organic compounds. They are defined by two Schiff's bases connecting three aromatic moieties. The central aromatic moiety typically features an *o*-phenylenediamine or its analog and two outer aromatic moieties salisaldehydes. They display potent binding capabilities with transition metal elements and are closely related to salens containing Schiff's bases that are constituted of aliphatic diamines [20]. Simple water-soluble lanthanum complexes are effective at detecting neutral sugars as well as glycolipids and phospholipids. The fluorescent lanthanum complex binds in solutions at physiologically relevant pH neutral sugars with apparent binding constants comparable to those of arylboronic acids [21].

The second aim of our study was to apply the developed OT-CEC method with salophene–lanthanide–Zn^{2+} complex-based stationary phase to the separation of glycated peptides (tryptic digest of BSA glycated by reactive oxocompounds, glucose and ribose). Glycation, *i.e.* non-enzymatic glycosylation (modification of proteins/peptides by saccharides), significantly affects the properties of peptides and proteins [22] and it is of great importance from a physiological point of view. Peptide mapping of glycated proteins by CZE in bare FS capillary is very difficult because of the presence of the relatively large cross-linked peptides in protein hydrolyzates, which strongly tend to stick to the capillary wall. Hence, the development and application of a new capillary coating (stationary phase), which could suppress the adsorption of these tryptic peptides and improve their separability is very desirable and challenging. Moreover, it could be potentially used for monitoring and investigation of glycation of proteins.

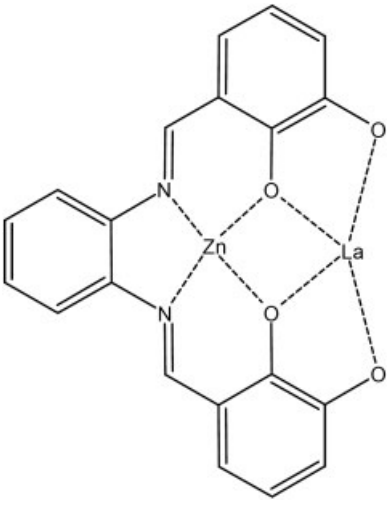

Figure 1. Structure of salophene–lanthanide–Zn^{2+} complex.

2 Materials and methods

2.1 Chemicals

Sodium dihydrogen phosphate, sodium tetraborate pentahydrate, zinc(II) sulfate, hydrochloric acid and dichloromethane were produced by Lachema (Brno, Czech Republic) and were of analytical grade quality. Methanol and EDTA (disodium salt) were obtained from Merck (Darmstadt, Germany), guanidine hydrochloride was from AppliChem (Darmstadt, Germany); iodoacetic acid, D,L-DTT, crystallized and lyophilized trypsin, ammonium bicarbonate, Tris-HCl, glucose and ribose were purchased from Sigma (St. Louis, MO, USA). The salophene–lanthanide–Zn^{2+} complex (Fig. 1) was synthesized by V. Král at the Department of Analytical Chemistry, Institute of Chemical Technology, Prague, for details see [21]. All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA) and filtered using syringe filter (Whatman, Clifton, NJ, USA), 0.45 μm pore size.

2.2 Glycation of BSA

BSA was dissolved in 0.2 M sodium phosphate buffer, pH 7.4, at a concentration of 1 mg/mL and incubated with the studied oxo-compounds, glucose and ribose, at 37°C for 96 h. The concentration of all reactants was 100 mM. BSA incubated under the same conditions in the buffer alone served as a control. After incubation, the samples were desalted by Econo-Pac 10 DG columns (Bio-Rad Laboratories, Richmond, CA, USA)

2.3 Reduction, alkylation and tryptic digestion of native and glycated BSA

BSA samples were dissolved in 0.5 mL of 6.0 M guanidine · HCl, 1.2 M Tris-HCl, 2.5 mM Na_2EDTA (pH 8.4) buffer, their disulfide bridges were reduced by adding 25 μL of 1.0 M DTT and placing at 65°C for 30 min. *S*-Carboxymethylation (alkylation) was carried out by adding 60 μL of 1.0 M iodoacetic acid and placing in the dark at room temperature for 40 min. The alkylation was stopped by adding 15 μL of 1.0 M DTT and then the samples were desalted and transferred to the digestion buffer by applying the reaction mixture to Econo-Pac 10 DG columns equilibrated with 20 mM NH_4HCO_3, pH 7.8, digestion buffer. The carboxymethylated proteins were eluted and collected with the digestion buffer. The treated proteins

were digested by incubation with trypsin at 1:50 enzyme/substrate ratio at 37°C for 3 h. Blank samples were prepared by incubating the enzyme solution alone under identical conditions. The samples were then centrifuged for 5 min at $2000 \times g$ and the supernatants removed and stored at −18°C.

2.4 Preparation of capillary stationary phase

A new FS capillary was sequentially washed with water, 1 M HCl, water, 1 M NaOH and water, each for 5 min. Next, it was washed with methanol, dichloromethane and dried by a stream of air. The capillary was then filled with a solution of the stationary-phase constituent (salophene–lanthanide–Zn^{2+} complex) in methanol (2 mg/mL) and deposited in an oven, where it was heated overnight at 40°C. After this procedure, the capillary was washed with the mobile phase for 5 min. To stabilize and equilibrate the stationary phase, the capillary was run in the mobile phase at 10 kV for 90 min, this procedure was repeated until baseline drift was eliminated.

2.5 CZE and OT-CEC

All CZE and OT-CEC runs were performed in Beckman P/ACE system 5500 (Beckman, Fullerton, CA, USA). A bare FS capillary with outer polyimide coating (Polymicro Technologies, Phoenix, AR, USA), 37 cm total length, 30 cm effective length to the detector, 50/375 μm id/od, was used for CZE separations. For OT-CEC analyses, this capillary was coated by salophene–lanthanide–Zn^{2+} complex-based stationary phase (Section 2.4). Detection was performed by UV-absorption detector operating at 214 nm. The sample was injected electrokinetically at 10 kV for 5 or 10 s. The separations were run at 25°C with an applied separation voltage of 10 kV. Prior to each analysis, capillary was washed with mobile phase for 5 min.

Table 1. Composition of mobile phases applied in CZE and OT-CEC separations of tryptic peptides of BSA

Mobile phase no.	$c_{Na_2B_4O_7}$ (mM)[a)]	c_{ZnSO_4} (mM)[b)]	pH
1	100	0	9.0
2	100	1.0	9.0
3	100	1.0	8.5
4	100	1.0	9.5
5	50	1.0	9.0
6	150	1.0	9.0
7	100	0.2	9.0
8	100	0.5	9.0
9	100	1.0	9.0
10	100	2.0	9.0

a) Concentration of sodium tetraborate
b) Concentration of zinc(II) sulfate.

A numerous series of mobile phases have been tested during the development of the CZE and OT-CEC methods and their composition is summarized in Table 1. In addition to mobile phases summarized in this table, two other mobile phases have been used in the early screening of tryptic peptides separations: 100 mM sodium phosphate, 1 mM $ZnSO_4$, pH 2.5, and 150 mM ammonium acetate, 1 mM $ZnSO_4$, pH 9.0. Because of poor separations in these two mobile phases, they were omitted in further developments. In all mobile phases used, pH was adjusted by 1.0 M HCl. Stability of column was at least 5 days.

3 Results and discussion

3.1 Development of separation conditions

The first step in the development of any analytical procedure is testing and optimization of experimental conditions. In this study, tryptic fragments of native BSA were used as a model polypeptide mixture for method development, and then the developed method was applied to the monitoring of BSA glycation by reactive oxocompounds, glucose and ribose. The initial screening of CZE separation of BSA tryptic peptides was carried out within a broad pH range of mobile phases from highly acidic (pH 2.5) *via* moderately acidic (pH 6.0) to weakly alkaline (pH 9.5), using phosphate, acetate and tetraborate buffers as mobile phase constituents, respectively. The separation of peptides in phosphate- and acetate-based mobile phases was rather poor (data not shown) and much worse than that in the tetraborate mobile phase; hence, only sodium tetraborate buffers at different concentrations (50, 100 and 150 mM), pH (8.5, 9.0 and 9.5) and without and with the addition of variable concentration of $ZnSO_4$ (0.2, 0.5, 1.0 and 2.0 mM) were tested as mobile phases in further method development. From these tests, the 100 mM sodium tetraborate, pH 9, with the addition of 1.0 mM $ZnSO_4$, was evaluated as the best choice.

Figure 2 shows the CZE separations of tryptic fragments of the native BSA in bare FS capillary in tetraborate mobile phase in the absence of Zn^{2+} ions (Fig. 2A) and in the presence of 1 mM Zn^{2+} ions, added in the form of $ZnSO_4$, (Fig. 2B). As follows from the comparison of these two separations, addition of Zn^{2+} ions into the mobile phase had a positive effect on the separation of these peptides, *i.e.* more peptides could be resolved with a higher resolution. This effect can be only partially ascribed to longer migration times of peptides due to the lower EOF; some changes of selectivity of separation can be observed as well due to the specific interactions of some peptides with Zn^{2+} ions in the mobile phase. Addition of 1 mM concentration of Zn^{2+} ions to the mobile phase was found as optimal also for the separation of the above tryptic peptides of BSA by OT-CEC in the FS capillary non-covalently coated with salophene–lanthanide–Zn^{2+} complex, see Fig 2C. It is worth to mention that addition of Zn^{2+} ions into the mobile phase was absolutely necessary since in the absence of Zn^{2+} ions in the mobile

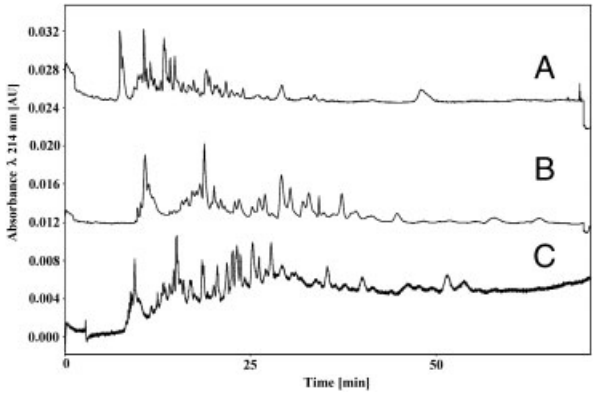

Figure 2. CZE and OT-CEC separations of tryptic peptides of native BSA. (A) CZE in bare FS capillary, mobile phase: 100 mM sodium tetraborate, pH 9.0; (B) CZE in bare FS capillary, mobile phase: 100 mM sodium tetraborate, pH 9.0, with addition of 1 mM $ZnSO_4$; (C) OT-CEC in FS capillary with salophene–lanthanide–Zn^{2+} complex stationary phase, mobile phase: 100 mM tetraborate, pH 9.0, with addition of 1 mM $ZnSO_4$. For other experimental conditions see Section 2.5.

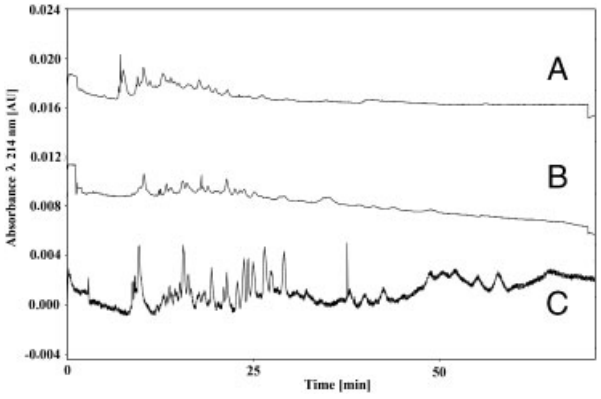

Figure 3. CZE and OT-CEC separations of tryptic peptides of glucose-glycated BSA. (A) CZE in bare FS capillary, mobile phase: 100 mM sodium tetraborate, pH 9.0; (B) CZE in bare FS capillary, mobile phase: 100 mM sodium tetraborate, pH 9.0, with addition of 1 mM $ZnSO_4$; (C) OT-CEC in FS capillary with salophene–lanthanide–Zn^{2+} complex stationary phase, mobile phase: 100 mM tetraborate, pH 9.0, with addition of 1 mM $ZnSO_4$. For other experimental conditions see Section 2.5.

phase the coating was unstable and the baseline was drifting, probably due to the leakage of Zn^{2+} ions from the complex. Apparently, the interactions of BSA tryptic peptides with the salophene–lanthanide–Zn^{2+} complex-based stationary phase resulted in further substantial improvement of their separations, a higher number of peaks could be distinguished and a higher separation efficiency was achieved providing a better resolution of separated peptides. It is reasonable to assume that this improvement of the separation was caused not only by the reduced adsorption of peptides to the inner FS capillary wall but also by the specific interactions of some BSA tryptic peptides with the salophene–lanthanide–Zn^{2+} complex, resulting in improving selectivity of the OT-CEC separation of this peptide mixture over the CZE separation of this mixure in the bare FS capillary.

3.2 CZE and OT-CEC separations of tryptic peptides of glycated BSA

In the second part of this study, the optimized CZE and OT-CEC experimental conditions were applied to the separation of tryptic peptides of glycated BSA. For the BSA glycation, two compounds possessing oxo-groups, glucose and ribose, have been applied. Glycation significantly affects the properties of peptides and proteins and it is of great importance from a physiological point of view. Analysis of peptides arising from tryptic cleavage of glycated proteins is quite difficult because the relatively large cross-linked polypeptides are frequently released with the increased tendency to stick to the bare FS capillary wall. The enhanced sorption of peptides arising from tryptic cleavage of glycated BSA is shown in Figs. 3 and 4, presenting CZE and OT-CEC separations of tryptic peptides of BSA glycated by glucose and ribose, respectively. CZE separations of these peptide

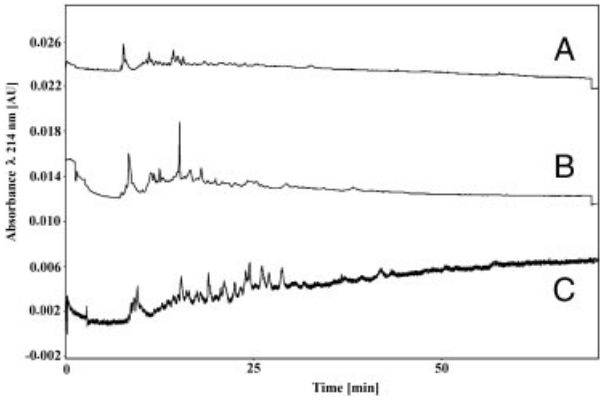

Figure 4. CZE and OT-CEC separations of tryptic peptides of ribose-glycated BSA. (A) CZE in bare FS capillary, mobile phase: 100 mM sodium tetraborate, pH 9.0; (B) CZE in bare FS capillary, mobile phase: 100 mM sodium tetraborate, pH 9.0, with addition of 1 mM $ZnSO_4$; (C) OT-CEC in FS capillary with salophene–lanthanide–Zn^{2+} complex stationary phase, mobile phase: 100 mM tetraborate, pH 9.0, with addition of 1 mM $ZnSO_4$.

mixtures in tetraborate mobile phases both in the absence and in the presence of Zn^{2+} ions (Figs. 3A and B and 4A and B) are much worse than the CZE separations of tryptic peptides of native BSA shown in Figs. 2A and B. Unlike bare FS capillary, in the capillary coated with salophene–lanthanide–Zn^{2+} complex, the sorption of tryptic peptides of glycated BSA is significantly reduced and *via* the interactions of this sugar-specific agent with some (glyco)-peptides, rather good OT-CEC separations (peptide maps) of tryptic hydrolysates of glucose and ribose-glycated BSA are obtained, as shown in Figs. 3C and 4C, respectively. This observation is in agreement with the previously published usability of lanthanide complexes as indicators for the binding of neutral sugars [21].

The comparison of the OT-CEC separations of tryptic peptides of native BSA and BSA glycated by glucose and ribose in FS capillary modified by salophene–lanthanide–Zn^{2+} complex-based stationary phase is shown in Fig. 5. The obtained OT-CEC-UV profiles (peptide maps) of the tryptic hydrolyzates of glycated BSA (Figs. 5B and C) unambiguously highlight the chemical and structural changes in BSA caused by its reaction with the oxo-compounds and they significantly differ from the profile of tryptic peptides of native BSA (Fig. 5A). The differences in electrochromatograms are manifested by the different numbers of the resolved peaks as well as by the changes of particular peak heights and areas. In the OT-CEC separation of tryptic peptides of native BSA (Fig. 5A), about 44 peaks could be distinguished, whereas in the separations of tryptic peptides of glycated BSA by glucose and ribose the number of resolved peaks was lower: 38 peaks were found in peptides map of BSA glycated by glucose (Fig. 5B) and only 30 peaks were resolved in the peptide map of BSA glycated by ribose (Fig. 5C), *i.e.* the number of peaks was reduced by about 14% after BSA modification by glucose and by about 32% after BSA modification by ribose. Thus, as expected, considerably more distinct changes were found after the modification of BSA with a more reactive oxo-compound (ribose) than by less reactive glucose. The milder modification of BSA by glucose indicates that with this agent presumably only monofunctional derivatives were formed. These observations are in agreement with our previously published results on glycation of BSA obtained by HPLC/MS study [23].

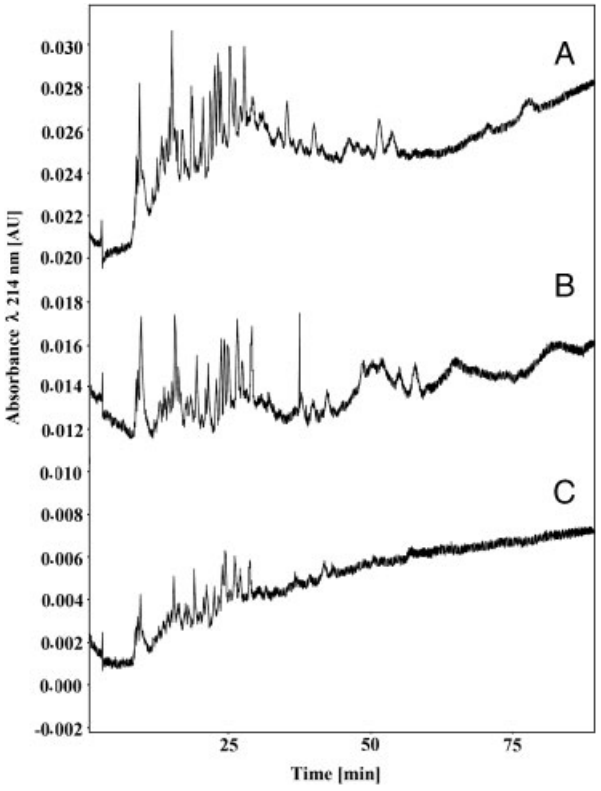

Figure 5. Comparison of OT-CEC separations of tryptic peptides of native and glycated BSA in FS capillary with salophene–lanthanide–Zn^{2+} complex stationary phase, mobile phase: 100 mM tetraborate, pH 9.0, with addition of 1 mM $ZnSO_4$. (A) Tryptic peptides of native BSA; (B) tryptic peptides of glucose-glycated BSA; (C) tryptic peptides of ribose-glycated BSA. For other experimental conditions see Section 2.5.

4 Concluding remarks

In this study, the separability of tryptic peptides of native and glycated BSA was much improved when their CZE separation in bare FS capillary was replaced by OT-CEC separation in the FS capillary coated with a new stationary phase, salophene–lanthanide–Zn^{2+} complex. The best separations were obtained with mobile phase composed of 100 mM sodium tetraborate buffer, pH 9, with the addition of 1 mM Zn^{2+} ions. Significant differences have been found in the OT-CEC-UV profiles of tryptic hydrolysates of native and glycated BSA, which differed by the different numbers of the resolved peaks as well as by the changes of particular peak heights and areas. Reduced peak numbers have been observed in the peptide maps of glycated BSA than in the native BSA. As expected, considerably more distinct changes were found after BSA modification with ribose as a more reactive oxo-compound than with glucose. Thus, the developed OT-CEC possesses a potential to be used for monitoring and investigation of protein glycation.

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