Journal of Chromatography B, 878 (2010) 2933–2936

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Capillary electrophoresis with noncovalently bilayer-coated capillaries for stability study of allergenic proteins in simulated gastrointestinal fluids

Chang Zheng, Youping Liu, Qiuhong Zhou, Xin Di*

School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, PR China

ARTICLE INFO

Article history:
Received 10 June 2010
Accepted 17 August 2010
Available online 26 August 2010

Keywords:
Capillary electrophoresis
Capillary coating
Allergenic proteins
Simulated gastrointestinal fluids

ABSTRACT

A novel noncovalently bilayer-coated capillary using cationic polymer polybrene (PB) and anionic polymer (sodium 4-styrenesulfonate) (PSS) as coatings was prepared. This PB–PSS coating showed good migration-time reproducibility for proteins and high stability in the range of pH 2–10 and in the presence of 1 M NaOH, acetonitrile and methanol. Capillary electrophoresis with PB–PSS coated capillaries was successfully applied to quantitatively investigate the stability of bovine serum albumin, ovomucoid, β-lactoglobulin and lysozyme in simulated gastrointestinal fluids. β-lactoglobulin A and β-lactoglobulin B were both stable in simulated gastric fluid with degradation percentages of 34.3% and 17.2% after 60 min of incubation, respectively. Bovine serum albumin, ovomucoid and lysozyme were stable in simulated intestinal fluid with degradation percentages of 17.7%, 23.4% and 22.8% after 60 min of incubation, respectively. The superiority of the proposed method over sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and capillary electrophoresis with untreated fused silica capillaries was demonstrated and emphasized.

1. Introduction

Food allergies are primarily the result of immune responses to food proteins [1]. Digestion resistance of a food protein is considered as an important parameter for its allergenic potential. Protein stability in simulated gastrointestinal fluids is believed to be related to resistance to proteolytic processes that are encountered within the digestive system and/or the intracellular environment [2–4]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is one of the analytical tools widely used for *in vitro* digestibility study of food proteins [5–8]. However, SDS-PAGE not only is time-consuming and tedious, but also fails to give accurate quantitative information. In addition, polyacrylamide used as a matrix for gel electrophoresis is a known neurotoxin. With the advantages of high efficiency, short analysis time and ease of automation, the potential of capillary electrophoresis (CE) for protein analysis is being recognized and demonstrated [9–11]. However, separation of proteins on a bare capillary is not satisfied due to the adverse interactions (mainly electrostatic and hydrophobic interactions) of protein molecules with silanol groups on the inner wall of the fused silica capillary. Adsorption of proteins usually leads to changes in the electroosmotic flow (EOF) and thus to a poor reproducibility of migration time as well as losses in peak efficiency. Recently, Maier et al. [12] reported a simple and fast CE method for quantitatively monitoring peptic digestibility of β-lactoglobulin isolated from different bovine milk products. In order to minimize possible protein adsorption onto the bare silica capillary wall, 0.05% Tween 20 was added to the background electrolyte (BGE). But no information was presented on the reproducibility of migration time and peak area of β-lactoglobulin.

It should be noted that evaluation of the reproducibility of CE assay is of crucial importance for tracking protein digestion. In order to achieve reliable analytical results of protein analysis by CE, various approaches have been explored to prevent the protein–wall interactions [13–16]. The use of capillary surface coating is one of the efficient methods to avoid the adsorption of proteins. A simple concept of capillary coating is by treating the capillary with one or more charged polymers that are physically adsorbed to the internal wall. Using this noncovalent-coating concept, some bilayers (e.g., PB-DS, PB-PVS, PDADMAC-PSS) and triple layers (e.g., PB-DS-PB) with polymers of opposite charge being alternately adsorbed on each other have been prepared [11,16–24]. The use of bilayer polymeric coatings in silica capillaries, employing PB in the first layer, was introduced by Katayama et al. [11]. Compared to the bare silica capillaries, noncovalently coated capillaries exhibited higher stability and analytical reproducibility under extreme conditions. Several applications of noncovalent coatings for protein analysis have been demonstrated [21–23]. To the best of our knowledge,

* Corresponding author at: P.O. Box 54, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, PR China. Tel.: +86 24 2398 6269; fax: +86 24 2390 2539.
E-mail address: dixin63@hotmail.com (X. Di).

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doi:10.1016/j.jchromb.2010.08.027

capillary electrophoresis with noncovalently coated capillaries for stability study of food proteins in simulated gastrointestinal fluids has not been reported.

In the present work, a noncovalently bilayer-coated capillary with cationic polymer polybrene (PB) and anionic polymer (sodium 4-styrenesulfonate) (PSS) as coatings was developed for the first time. Using bovine serum albumin (BSA), ovomucoid (OVM), β-lactoglobulin (βLG) and lysozyme (Lys) as model proteins, the feasibility of capillary electrophoresis with PB–PSS coated capillaries for stability study of allergenic proteins in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was demonstrated.

2. Experimental

2.1. Materials and equipments

Polybrene and poly(sodium 4-styrenesulfonate) were purchased from Sigma–Aldrich. β-Lactoglobulin (CAT No. L0130), β-lactoglobulin B (CAT No. L8005) and ovomucoid (CAT No. T9253) were all purchased from Sigma–Aldrich. Lysozyme (CAT No. 64006060), bovine serum albumin (CAT No. 69003431), pepsin (CAT No. 64007137) and pancreatin (CAT No. 64006737) were from Sinopharm Chemical Regent Co., Ltd. (Shanghai, China). Insulin (28 IU/MG) was purchased from Wanbang Biopharma (Jiangsu, China). Tris (>99.8%) was from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Potassium dihydrogen phosphate and sodium tetraborate were both from Bodi Chemical Holding Co., Ltd. (Tianjin, China). N,N-dimethylformamide (DMF) was from Fuchen Corporation (Tianjin, China). Sodium hydroxide, hydrochloric acid and phosphoric acid of analytical grade were obtained from Dongxing Corporation (Shenyang, China), Xinyang Corporation (Henan, China) and Damao Corporation (Tianjin, China), respectively. Methanol and acetonitrile of HPLC grade were purchased from Concord Corporation (Tianjin, China). Bare fused silica capillaries of 50 μm i.d. and 375 μm o.d. were from Yongnian Optic Fiber Plant (Hebei, China).

2.2. PB–PSS coating procedure

The preparation of the noncovalent bilayer-coated capillary was as follows: a new bare fused silica capillary was left to stand filled with 1 M NaOH for 12 h followed by 10-min rinse with water. Coating was performed by flushing the capillary with 3% (m/v) PB aqueous solution for 20 min, leaving the capillary to stand for 15 min and then flushing with water for 5 min. Subsequently, the capillary was flushed with 1% (m/v) PSS aqueous solution for 20 min and left to stand for 15 min followed by flushing with water for 5 min. The PSS coating procedure was repeated twice. Before each run, the capillary was flushed with water, 0.1 M NaOH, water and BGE for 1, 1, 1 and 2 min, respectively. All the flushing steps were performed at about 940 mbar.

2.3. SGF or SIF digestion stability assay

BSA, OVM, βLG and Lys were chosen as the test proteins for digestion stability assay and were separately prepared at 1.0, 1.0, 2.0 and 1.0 mg/mL in water. SGF and SIF were freshly prepared as described in the United States Pharmacopoeia. For SGF, 2.0 g sodium chloride and 3.2 g pepsin were dissolved in 7.0 mL hydrochloric acid and a sufficient volume of water to make 1000 mL. The pH of SGF was approximately 1.2. For SIF, 6.8 g potassium dihydrogen phosphate was dissolved in 250 mL water. Then 77 mL 0.2 N sodium hydroxide and 500 mL water were added and mixed along with 10.0 g pancreatin. The SIF solution was adjusted to pH 6.8 ± 0.1 with either 0.2 N sodium hydroxide or 0.2 N hydrochloric acid and then diluted with water to 1000 mL.

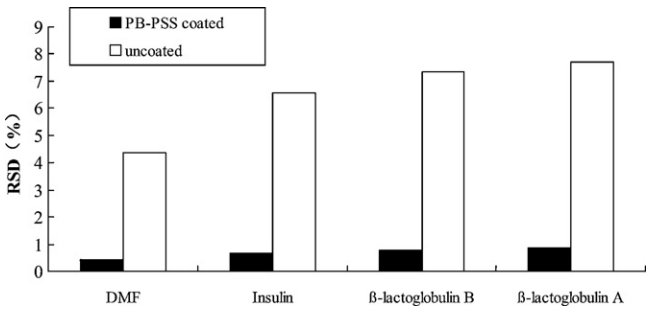

Fig. 1. Comparison of migration-time reproducibility for protein analysis by CE using a PB–PSS coated capillary and a bare fused silica capillary. Conditions: capillary, 50 μm i.d. × 55 cm (46.7 cm effective length); BGE, 400 mM Tris-H_3PO_4 at pH 7.5; applied voltage, 25 kV; detection wavelength, 220 nm; hydrodynamic injection, 35 mbar for 6 s.

A 100-μL aliquot of SGF or SIF was pipetted into a 1.5-mL microcentrifuge tube and incubated in a water bath at 37 °C for 5 min, and then 100 μL of test protein was added to start the reaction. At intervals of 0, 0.25, 2, 10, 30 and 60 min, 70 μL of 200 mM Na_2CO_3 (for SGF) or 1% (v/v) HCl (for SIF) was added to each tube to stop the reaction. The sample solutions obtained at each time interval were mixed carefully and cooled to room temperature directly for CE analysis.

2.4. CE instrumentation and conditions

CE analysis was carried out using an HP^{3D} CE system (Agilent Technologies, Waldbrom, Germany) equipped with a built-in photometric diode-array detector and controlled by the ChemStation CE software. Separation was performed on a PB–PSS bilayer-coated capillary with a total length of 33 cm (24.7 cm effective length). The temperature of the capillary cassette was kept at 25 °C. The running buffer and separation voltage for the analysis of BSA, OVM, βLG and Lys in SGF and SIF were listed in Table 1. The detection wavelength was set at 200 nm for BSA, OVM and Lys and 220 nm for βLG. Hydrodynamic injection was performed for 8 s at 50 mbar.

3. Results and discussion

3.1. Characteristics of PB–PSS coating

The stability of PB–PSS coating was investigated by measuring the EOF in a wide pH range. It was found that the coating was able to generate a stable EOF (3.51×10^{-4} to 4.43×10^{-4} $cm^2/V/s$) over the pH 2–10 range and was stable for at least 100 runs. The chemical stability of PB–PSS coating was studied by measuring the EOFs before and after rinsing with 1 M NaOH, CH_3OH, CH_3CN and 0.1 M HCl. The coating was found to be highly stable against 1 M NaOH, CH_3OH and CH_3CN, as the degradation ratios of EOF were all below 0.6%. However, the coating appeared to be slightly unstable after exposure to 0.1 M HCl with a degradation ratio of 4%.

The efficiency and reproducibility of PB–PSS coating for protein analysis was investigated using DMF (EOF marker), insulin and βLG as test compounds. Two isoforms A and B of βLG (i.e., βLGA and βLGB) were well separated and both plate numbers exceeded 4.7×10^5/m. The RSD of migration time for DMF was 0.26% for intraday ($n = 5$) and 0.87% for interday ($n = 5$). The RSDs of migration time for insulin, βLGA and βLGB were all less than 0.87% ($n = 5$), whereas the corresponding RSD was above 6.55% for bare fused silica capillary (Fig. 1). These results suggested that the PB–PSS coating effectively prevented adverse protein–wall interactions.

Table 1
CE conditions for test proteins in SGF and SIF digestion assay.

	CE conditions[a]	
	SGF digestion	SIF digestion
BSA	50 mM KH_2PO_4 pH 7.4 (10 kV)	50 mM KH_2PO_4 pH 8.0 (12 kV)
OVM	30 mM $Na_2B_4O_7$–HCl pH 8.5 (18 kV)	30 mM $Na_2B_4O_7$–HCl pH 8.5 (15 kV)
βLG	500 mM Tris–H_3PO_4 pH 7.4 (12 kV)	500 mM Tris–H_3PO_4 pH 7.4 (13 kV)
Lys	20 mM KH_2PO_4 pH 2.5 (20 kV)	20 mM KH_2PO_4 pH 2.5 (20 kV)

[a] CE conditions are as described in Section 2.4.

3.2. Stability of proteins in SGF and SIF

The repeatability of migration time and peak area for each test protein was checked by five replicate injections of SGF incubation solutions at 0 min. The RSDs were 0.43% and 1.60% for BSA, 0.17% and 0.65% for OVM, 1.48% and 2.07% for βLGA, 1.37% and 0.56% for βLGB, 0.46% and 1.32% for Lys, respectively. The repeatability of incubation process was evaluated by five replicate incubations of BSA, OVM and Lys in SIF for 60 min. The RSDs of migration time and peak area were 0.57% and 1.34% for BSA, 1.36% and 1.65% for OVM, 0.77% and 1.18% for Lys, respectively. These results indicated that the developed CE method could be applied to quantitatively investigate the stability of allergenic proteins in simulated gastrointestinal fluids.

Table 2 summarizes the digestibility of the test proteins in SGF and SIF. Degradation percentages of the test proteins which were stable in SGF or SIF for at least 60 min were determined by comparing the peak areas obtained at 0 min with those at 60 min.

In SGF digestion stability assays, BSA was observed to be rapidly digested within 0.25 min at the beginning of incubation time and some fragments were formed. βLGA and βLGB were both stable in SGF with slow decrease in peak area. The degradation percentages of βLGA and βLGB were 34.3% and 17.2% at the final time point (60 min) indicating that the amount of intact βLGB decreased slower than that of βLGA. Significant peak broadening and abnormal peak shape were observed for OVM after 0.25 min of incubation in SGF. Lys was also less stable in SGF than in SIF and completed degradation was obtained after 60 min of incubation in SGF. From the results above, it is observed that 3 out of 4 tested proteins have low resistance to pepsin, indicating the importance of pepsin for food protein digestion.

In SIF digestion stability assays, BSA, OVM and Lys were more stable than in SGF. Fig. 2 shows the degradation process of OVM in SIF at several increasing time intervals over 60 min. It was found that a minor peak appeared in a region between the matrix peak and OVM peak after 30 min of incubation. This observation indicates that OVM was partially degraded in SIF within 60 min. With SDS-PAGE method, no degradation product was observed [6]. Fig. 3 shows the degradation process of βLGA and βLGB in SIF. It can be seen that both were completely degraded in SIF within 10 min. By comparison, the degradation rate of βLGB was faster than βLGA. Moreover, many peaks corresponding to their degradation products were observed in the electrophoregram after 2 min

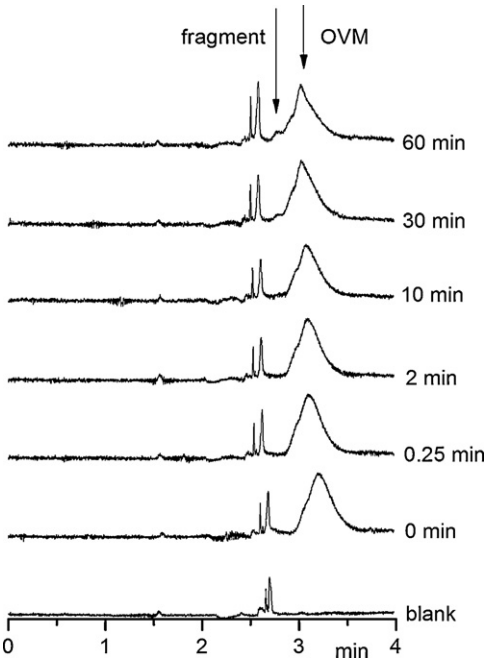

Fig. 2. CE analysis of SIF digestion stability of ovomucoid. Conditions: capillary, 50 μm i.d. × 33 cm (24.7 cm effective length); BGE, 30 mM $Na_2B_4O_7$–HCl at pH 8.5; applied voltage, 15 kV; detection wavelength, 200 nm; hydrodynamic injection, 50 mbar for 8 s.

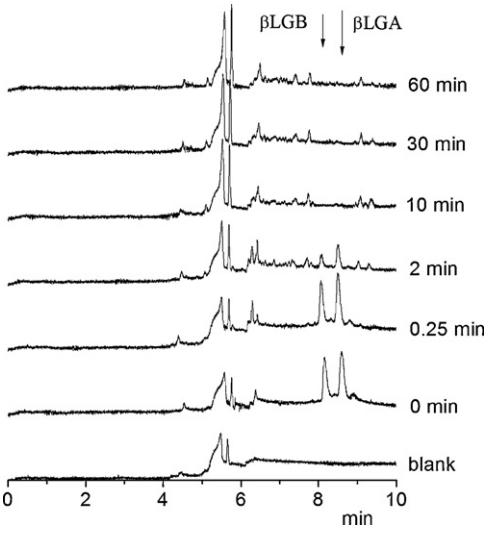

Fig. 3. CE analysis of SIF digestion stability of β-lactoglobulin A and B. Conditions: capillary, 50 μm i.d. × 33 cm (24.7 cm effective length); BGE, 500 mM Tris–H_3PO_4 at pH 7.4; applied voltage, 13 kV; detection wavelength, 220 nm; hydrodynamic injection, 50 mbar for 8 s.

Table 2
Digestibility of test proteins in SGF and SIF calculated as last time point that protein could be observed in CE electrophoregram.

Protein	Source	SGF stability (min)	SIF stability (min)
BSA	Cow's milk	0	60 (17.7%)[a]
βLGA	Cow's milk	60 (34.3%)[a]	2
βLGB	Cow's milk	60 (17.2%)[a]	2
OVM	Egg	0	60 (23.4%)[a]
Lys	Egg	30	60 (22.8%)[a]

[a] The number in parentheses is the degradation percentage of protein at the last time point.

of incubation, whereas only one fragment band was found in the SDS-PAGE gel [6].

In our study, new information on digestibility of OVM, βLGA and βLGB was obtained as described above. Moreover, with the present method, βLGA and βLGB were well separated both in SGF and SIF while baseline separation of βLGA and βLGB could not be achieved using CE with untreated fused silica capillary as reported by Maier et al. [12]. The previously published SDS-PAGE methods also failed to separate βLGA and βLGB [6,7]. OVM was completely separated from pepsin and pancreatin under the current electrophoretic condition; whereas the pepsin band interfered with a broad OVM band under the SDS-PAGE conditions [6,8]. These observations indicated that the separation power of our method was superior to the previously used methods to study the digestibility of these proteins in SGF and SIF.

4. Conclusions

This is the first report describing the use of capillary electrophoresis with noncovalently bilayer-coated capillary for stability study of food proteins in simulated gastrointestinal fluids. The preparation of the proposed PB–PSS coating is simple and straightforward. The test proteins, BSA, OVM, βLG and Lys, can be successfully separated from pepsin and pancreatin with high reproducibility of migration time and peak area, indicating the possibility of using PB–PSS coated capillary to quantitatively study the stability of allergenic proteins in simulated gastrointestinal fluids. It should be noted that resistance to SGF or SIF is not a sufficient criterion for evaluating food allergen sensitization or induction. The assessment of food allergen requires the use of both digestion and immunology assays as useful means to ensure consumer safety to food proteins. In future studies, the present analytical protocols will be applied for the evaluation of the persistence and resistance to digestion of food proteins that could be new allergenic proteins, based on similarities to known protein allergens. Moreover, the obtained data from *in vitro* digestion assay will be correlated with information about immunological antigen–antibody reaction from the bibliography.

Acknowledgements

The present study was supported by a Key Project for Drug Innovation (2009ZX09301-012) from the Ministry of Science and Technology of China and a Research Grant (No. 2008711) from the Provincial Education Department of Liaoning.

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